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

Eleni Karinou[1^], Christopher F. Schuster[1&], Manuel Pazos[2&], Waldemar Vollmer[2] and Angelika Gründling[1*]

[1]Section of Microbiology and MRC Centre for Molecular Bacteriology and Infection, Imperial College London, London SW7 2AZ, UK

[2]The Centre for Bacterial Cell Biology, Newcastle University, Newcastle upon Tyne NE2 4AX, UK

^ Present address: The Centre for Bacterial Cell Biology, Newcastle University, Newcastle upon Tyne NE2 4AX, UK

& these authors contributed equally

* For correspondence:

Angelika Gründling

Section of Microbiology and MRC Centre of Molecular Bacteriology and Infection

Flowers Building RM 3.21

Imperial College London; London, SW7 2AZ, UK

Tel. +44 (0)2075945256

Fax +44 (0)2075943095

E-mail: a.grundling@imperial.ac.uk

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 4-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 sizable 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 (6).

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 (7). *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 (8). 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 (7). It is composed of glycan chains made of alternating N-acetylglucosamine and N-
74 acetylmuramic acid residues connected by peptide bridges (9). 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 (10). 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 (11). PBPs with glycosyltransferase and transpeptidase activity
79 polymerize the glycan chains and form peptide cross-bridges while the monofunctioal
80 transpeptidases only have the former activity (11).

81 *S. aureus* encodes four PBPs, of which PBP1, which has transpeptidase activity, and
82 the bifunctional PBP2, which has transpeptidase and glycosltransferase activity, are the
83 minimal requirement for cell survival (12). In methicillin-resistant *S. aureus* (MRSA) strains,
84 the alternative PBP2A, which has transpeptidase activity, is needed for β-lactam resistance in
85 addition to the glycosyltransferase activity of PBP2 (13, 14). Moreover, additional non-
86 essential proteins have been identified that are involved in peptidoglycan synthesis such as the
87 monofunctional glycosyltransferases SgtA and SgtB (also named Mgt) (12). Previous studies
88 have shown that although SgtA and SgtB have glycosyltransferase activity *in vitro*, only SgtB
89 can support the growth of *S. aureus* in the absence of the main glycosyltransferase PBP2.
90 However, this is not possible in the presence of β-lactam antibiotics when an interaction
91 between PBP2 and PBP2A is needed (15, 16).

92 In Gram-positive bacteria, the peptidoglycan layer is interspersed by a plethora of
93 proteins and cell wall polymers named teichoic acids (17). Teichoic acids are further
94 categorised into lipoteichoic acids (LTA), anchored to the outer leaflet of the cytoplasmic
95 membrane via a lipid moiety, and wall teichoic acids (WTA) covalently attached to the
96 peptidoglycan (18). Teichoic acids form an important part of the cell wall and contribute to the
97 physical and chemical properties of the cell wall, and to the binding of divalent cations (19,
98 20). While both WTA and LTA are polyanioinic cell wall polymers, they are synthesized
99 through separate and independent pathways in *S. aureus* and many other Gram-positive

100 bacteria (21, 22). Consistent with this, our work on the Gram-positive pathogen *Listeria*
101 *monocytogenes* has revealed that LTA synthesis is not abrogated in the absence of WTA and
102 *vice versa* (23). Recent work using pathway specific inhibitors and a gene interaction screen
103 provided further evidence that the polymers are not only synthesized through separate
104 pathways but also have distinct functions in *S. aureus* (24).

105 LTA is an anionic polymer made up in *S. aureus* of glycerolphosphate repeating units
106 that are further decorated with D-Alanine residues and, as shown recently, under high salt
107 conditions also with N-acetylglucosamine residues (25, 26). Most proteins required for its
108 synthesis have now been identified and extensively studied over the years (22, 27). One of the
109 key enzymes required for LTA synthesis is the lipoteichoic acid synthase enzyme LtaS (28,
110 29). This enzyme polymerizes the LTA backbone chain on the outside of the cell using the
111 glycerolphosphate head group of the membrane lipid phosphatidylglycerol as substrate (29,
112 30). LTA is indispensable for the growth of *S. aureus* under standard laboratory growth
113 conditions, highlighting its important physiological role (29, 31).

114 Previous studies indicated a function for LTA in helping to direct the cell-division
115 machinery (32), in controlling autolysin activity (31), for biofilm formation (33), in mediating
116 interactions with host cell receptors (17), in controlling susceptibility and/or resistance to
117 antimicrobial peptides and in maintaining cation homeostasis (20). *S. aureus ltaS* mutants,
118 which lack the complete LTA polymer, can in some strain backgrounds be constructed at low
119 growth temperature (31). It has also been shown that LTA-deficient *S. aureus* strains are viable
120 when grown under osmotically stabilizing conditions in medium containing 7.5% NaCl or 40%
121 sucrose (31, 34). However, the bacteria display severe morphological defects including an
122 enlarged cell size, clustering and cell division defects, even under those conditions permissive
123 for growth (34). Bacteria can also readily acquire compensatory mutations allowing them to
124 grow in un-supplemented medium and improving their cell morphology defects (34). The

125 majority of compensatory mutations previously observed were in *gdpP* leading to inactivation
126 of the c-di-AMP phosphodiesterase GdpP (34). The resulting increase in cellular c-di-AMP
127 levels allowed the bacteria to survive the cell wall stress caused by the absence of LTA, which
128 is now believed to be due to changes in the osmotic balance in the cell (34). However,
129 compensatory mutations that rescue the growth of an LTA negative *S. aureus* strain were not
130 only found in *gdpP* but also in other genes (34).

131 As part of the current study, we sought to identify these genes and further characterize
132 the encoded proteins to gain additional insight into why LTA is essential for the growth of *S.*
133 *aureus* and potentially uncover novel cellular functions for proteins involved in cell wall
134 assembly or maintenance. Using a suppressor screen approach, we found that inactivation of
135 the monofunctional peptidoglycan glycosyltransferase SgtB allows *S. aureus* to grow in the
136 absence of LTA. We further show that peptidoglycan crosslinking is significantly reduced in
137 the absence of LTA, which can be partially restored upon inactivation of SgtB. This in turn
138 might strengthen the peptidoglycan layer and, in this way, contribute to the observed growth
139 rescue.

140

141 **Results**

142 **Identification of *S. aureus* suppressor strains able to grow in the absence of LTA in a c-**
143 **di-AMP-independent way**. LTA-deficient *S. aureus* strains are viable when grown under
144 osmotically stabilizing conditions in medium containing 7.5% NaCl or 40% sucrose or in un-
145 supplemented medium after the acquisition of compensatory mutations (31, 34). The majority
146 of compensatory mutations previously observed were in *gdpP* leading to inactivation of the c-
147 di-AMP phosphodiesterase GdpP (34). In the same study, suppressor strains with mutations
148 outside the *gdpP* gene were noted (34). In order to characterize suppressor strains with
149 mutations in genes other than *gdpP* and to gain further insight into the cellular function of LTA,

150 a larger suppressor screen was performed and the *ltaS* mutant strains LAC*Δ*ltaS::erm*
151 constructed in medium supplemented either with sucrose or NaCl were plated on un-
152 supplemented TSA plates. A number of independently obtained suppressor colonies were
153 subsequently passed 4 times in fresh TSB to further improve their growth. Next, the
154 chromosomal DNA was isolated from 80 suppressor strains and those lacking mutations in
155 *gdpP* (coding for the c-di-AMP hydrolase) and *dacA* (coding for the c-di-AMP cyclase
156 enzyme) were identified by determining the sequences of these two genes. Of 80 colonies
157 screened, none had mutations in *dacA* and 17 strains had no mutation in *gdpP*, 7 of which were
158 selected for further analysis. The absence of LTA but presence of WTA in the suppressor
159 strains was confirmed by western blot and Alcian blue silver staining analysis, respectively
160 (Figs. 1A and 1B). Next, the relative cellular c-di-AMP levels in the different strains were
161 determined using a previously described competitive ELISA assay (35, 36) (Fig. 1C). In
162 contrast to the *gdpP* mutant or LTA negative suppressor strain with a mutation in *gdpP*, which
163 showed the expected increase in c-di-AMP levels, the 7 suppressor strains chosen for further
164 analysis did not show an increase in the cellular c-di-AMP concentration (Fig. 1C).
165
166 **Identification of genomic alterations in the *S. aureus* suppressor strains able to grow in**
167 **the absence of LTA.** Next, the genomic sequences of the seven suppressor strains were
168 determined and compared to that of the original *ltaS* mutant strains using a whole genome
169 sequencing approach. Mutations were found in *sgtB* (SAUSA300_1855), coding for the
170 monofunctional glycosyltransferase SgtB and mutations in this gene arose independently in
171 three suppressor strains (Table 1). Another strain had a mutation in SAUSA300_1254, coding
172 for a hypothetical membrane protein as well as SAUSA300_RS11150, encoding MazE, the
173 antitoxin component of a type II toxin-antitoxin module (Table 1). In the original study by
174 Corrigan *et al*. (34), mutations found in SAUSA300_1254 were proposed to be accessory and

175 required to further improve the growth of the *gdpP* mutant suppressor strains. Consistent with
176 a previous report (2), a large deletion in *clpX* (SAUSA300_1621), encoding a protein forming
177 part of an ATP-dependent protease, was observed in one strain (Table 1). Finally, a mutation
178 in *vraT* (SAUSA300_1867), coding for the membrane protein VraT and forming part of the
179 VraRST three-component system, was identified. Using our standard genome sequence
180 analysis workflow, an unusually large number (>300) of zero coverage regions were obtained
181 for suppressor strain S6, preventing us from matching a single nucleotide polymorphism with
182 high confidence to the observed growth rescue. Hence, suppressor stain S6 is not listed in Table
183 1 and was also not further analyzed. The mutations identified in the other suppressor strains
184 were subsequently confirmed by fluorescent automated resequencing of the respective genomic
185 region. Some of the mutations observed in *sgtB*, as well as the mutations in *vraT*, *clpX* and
186 *mazE* result in frameshift mutations and introduction of premature stop codons, suggesting that
187 the absence of the encoded proteins compensates for the lack of LTA.
188
189 **Phenotypic characterization of the LTA negative *S. aureus* suppressor strains**. In a
190 previous study, it was shown that *S. aureus* cells can grow without LTA in the absence of ClpX
191 (2), hence we did not further characterize the *clpX* mutant suppressor strain obtained as part of
192 this study. Instead our further analysis focused on suppressor strains with mutations in novel
193 genes, which are strains with mutations in *mazE*, *sgtB* or *vraT*. MazE, the antitoxin component
194 of a type II TA module, is part of the *sigB* regulon and has been shown to be essential for full
195 activity of the alternative sigma factor SigB (37). SigB and members of its regulon enable
196 bacteria to respond rapidly to environmental and antibiotic stresses and also play a role in cell
197 envelope homeostasis (38, 39). Various studies have investigated the role of the VraTSR three-
198 component regulatory system and this system has been reported to be involved in the induction
199 of the cell wall stressosome, mainly in the presence of cell wall targeting antibiotics (40-42).

200 Interestingly, the monofunctional glycosyltransferase SgtB, also identified as part of our
201 screen, is one of the proteins belonging to the cell wall stressosome whose expression is
202 regulated by VraTSR independent of the presence of cell wall targeting antibiotics (40). To
203 further characterize the suppressor strains, we first confirmed the growth improvement of
204 strains S2-*mazE*, S4-*sgtB* and S7-*vraT* when propagated in TSB. All three suppressor strains
205 grew better compared to the original *ltaS* mutant strain and their growth rate was only slightly
206 reduced compared to the WT LAC* stain (Fig. 2A). Next, the cell morphology of the WT,
207 original *ltaS* mutant and the three suppressor strains was assessed by microscopy following
208 staining of the peptidoglycan with fluorescently labelled vancomycin. The cell morphology of
209 the suppressor strains was considerably improved and in particular for suppressor strain S2-
210 *mazE* the division site was correctly placed in most cells (Fig. 2B). Next, the susceptibility of
211 the suppressor strains to a number of cell wall targeting antibiotics was determined. Although
212 the growth and cell morphology of the suppressor strains were improved, these strains
213 remained hyper-sensitive to the β-lactam antibiotic oxacillin and the MIC was reduced by 32-
214 fold or more for the different suppressor strains as compared to the WT LAC* strain (Fig. 3A).
215 The susceptibility to the cell wall or membrane targeting antibiotics lysostaphin, nisin,
216 vancomycin or daptomycin was also tested but no drastic differences were observed (Table 2).
217 Finally, the growth of the three different suppressor strains was assessed on plates containing
218 Congo red. Congo red is an anionic azo dye traditionally used for the detection of biofilms in
219 *Staphylococcus*. However, at higher concentrations it inhibits the growth of *S. aureus* and has
220 been used in the past to indicate differences in the cell wall integrity of different *S. aureus*
221 strains (43). Recently, the target of Congo red has been established as the LTA synthase
222 enzyme LtaS (44). Hence our suppressor strains, which are deleted for *ltaS* and are able to grow
223 in the absence of LTA, should be resistant to this dye. To test the susceptibility of the
224 suppressor strains, serial dilutions of overnight cultures were spotted on TSA plates containing

225 0.1% Congo red (Fig. 2C). The suppressor strains carrying mutations in *mazE* and *sgtB* were
226 significantly more resistant to Congo red than the WT strain, indicating that inactivation of
227 either one of these genes is indeed sufficient to bypass the LTA essentiality. However, the
228 suppressor strain with the mutation in *vraT* grew poorly on the Congo red plates, suggesting
229 that this strain might not be a bona fide suppressor strain.
230
231 **Introduction of SgtB or MazE in the respective suppressor strain results in the expected**
232 **growth arrest**. The result of the whole genome sequencing analysis suggested that inactivation
233 of MazE or SgtB is sufficient to allow *S. aureus* to grow in the absence of LTA. Introduction
234 of a wild-type copy of *mazE* or *sgtB* into the respective suppressor strain should restore this
235 phenotype and be lethal for the suppressor strains when grown in un-supplemented medium
236 but should not have an effect when the bacteria are propagated in medium supplemented with
237 40% sucrose. In order to test this, plasmids piTET-*mazE* or piTET-*sgtB,* allowing for
238 anhydrotetracycline (Atet) inducible gene expression, were introduced into the respective
239 suppressor strains. As control, these plasmids were also introduced in the WT LAC* strain.
240 Serial dilutions of these different strains were spotted onto TSA plates containing 100 ng/ml
241 or 200 ng/ml Atet for the expression of *mazE* or *sgtB* expression, respectively. As expected for
242 a successful complementation, the expression of *mazE* prevented the growth of the suppressor
243 strain S2-*mazE* (Fig. 4A) and the expression of *sgtB* prevented the growth of the suppressor
244 strain S4-*sgtB* (Fig. 4B) on TSA but had no effect when the bacteria were spotted on medium
245 supplemented with 40% sucrose (Fig. 5). Of note, the *mazE* suppressor strain S2-*mazE*
246 contained an additional mutation in a gene coding for a membrane protein of unknown function
247 with locus tag SAUSA300_125. Introduction of a WT copy of this gene in the suppressor strain
248 did not prevent the growth of this suppressor strain (data not shown). Taken together, the results

249 of this complementation analysis support the notion that inactivation of SgtB or MazE is
250 sufficient to allow *S. aureus* to grow in the absence of LTA.
251
252 **Growth characterization and antibiotic resistance of an *S. aureus* LAC* *sgtB* mutant**.
253 Inactivation of MazE likely has pleotropic effects due to its involvement in the activity of the
254 alternative sigma factor SigB and the reason as to why its inactivation allows *S. aureus* to grow
255 in the absence of LTA could be indirect. Hence, we next focused in trying to gain a better
256 understanding of the cellular function of the monofunctional peptidoglycan glycosyltransferase
257 SgtB and how its inactivation allows *S. aureus* to survive in the absence of LTA. To this end,
258 the *sgtB* mutant strain LAC**sgtB::tn* was constructed by moving the genomic region with a
259 transposon insertion in *sgtB* from the Nebraska transposon mutant library strain NE596 (45)
260 into the LAC* background. SgtB is one of two monofunctional peptidoglycan
261 glycosyltransferases encoded in the *S. aureus* genome and the protein can polymerize
262 peptidoglycan glycan chains *in vitro* (46). Although it is dispensable for the growth of *S.*
263 *aureus*, SgtB becomes necessary for bacterial survival in the absence of the main
264 glycosyltransferase PBP2 (12, 15, 47). Consistent with these previous observations, no
265 difference was observed in the cell growth and morphology of the *sgtB* mutant *S. aureus* strain
266 LAC**sgtB::tn* as compared to the WT LAC* strain (Fig. 6A and 6B). Our results indicate that
267 inactivation of SgtB allows *S. aureus* to survive in the absence of LTA, therefore the *sgtB*
268 mutant strain should no longer be sensitive to the Congo red dye. To test this, serial dilutions
269 of the WT, the *sgtB* mutant and a complementation strain were spotted on TSA plates
270 containing 0.1% Congo red, which inhibits the LtaS enzyme. Indeed, the *sgtB* mutant strain
271 was considerably more resistant to Congo red than the WT strain and this phenotype could be
272 complemented by introducing a functional copy of *sgtB* into the mutant strain (Fig. 6C). Next,
273 we also tested the susceptibility of the *sgtB* mutant and complementation strain towards the

274 cell wall active antibiotics oxacillin, lysostaphin, nisin, vancomycin and daptomycin. While no
275 differences were observed in the susceptibility of the *sgtB* mutant compared to a wild-type
276 strain towards most antibiotics, a slight (4-fold) and statistically significant increase in
277 resistance towards oxacillin was observed (Fig. 3B, Table 2). Taken together, while no growth,
278 or clearly visible morphological differences were observed between the WT and the *sgtB*
279 mutant strain, deletion of *sgtB* leads to increased Congo red dye and 4-fold increased oxacillin
280 antibiotic resistance in our strain background.
281
282 **Inactivation of SgtB leads to an increase in peptidoglycan cross-linking in an LTA-**
283 **negative *S. aureus* strain**. Since SgtB is involved in peptidoglycan synthesis and the *sgtB*
284 mutant displayed increased slightly increased oxacillin resistance, we hypothesised that its
285 deletion could somehow "strengthen" the cell wall through alterations in the peptidoglycan
286 structure. In order to investigate this, the muropeptide profiles of peptidoglycan isolated from
287 the WT LAC*, the *sgtB* mutant strain LAC**sgtB::tn*, and the *ltaS/sgtB* mutant suppressor strain
288 S4-*sgtB* were determined following the growth of these strains in TSB. In addition, the
289 muropeptide profiles were also determined for peptidoglycan isolated from these three strains
290 as well as the original *ltaS* mutant strain following growth in TSB supplemented with 40%
291 sucrose (Fig. 7). The WT and *sgtB* mutant strains showed very similar and typical *S. aureus*
292 muropeptide profiles (Fig. 7A). The chemical structure of several muropeptide fragments for a
293 number of these peaks has been previously determined in the seminal paper by de Jonge *et al.*
294 (48). We numbered the different peaks where possible as described in de Jonge *et al.* (48) with
295 peaks 1-5 being monomeric, 9 -14 dimeric, 15 trimeric and 16 and above higher oligomeric
296 muropeptide fragments. Quantification of the monomeric, dimeric, trimeric and higher
297 oligomeric peptidoglycan fragments showed that the peptidoglycan is highly crosslinked in
298 both strains with approximately 70% of the UV absorbing material found in the higher

299 oligomeric fraction (Fig. 7B and 7C). No clear differences were found between the
300 muropeptide profiles of the WT and *sgtB* mutant strain, either grown in TSB or TSB
301 supplemented with 40% sucrose (Fig. 7). In contrast to the WT and *sgtB* mutant strain, a
302 decrease in the higher-oligomeric peptidoglycan material was observed for the *ltaS* suppressor
303 strain S4-*sgtB* both in TSB and TSB sucrose medium (Fig 7). A visual inspection of the
304 chromatograms also indicated that peak 12, corresponding to a currently unknown
305 muropeptide, was reduced in *S. aureus* strains unable to produce LTA, which are the original
306 *ltaS* mutant as well as the *S4-ltaS* suppressor strain (Fig. 7A). But perhaps most notably, a
307 comparison of the muropeptide profiles of the peptidoglycan isolated from the original *ltaS*
308 mutant and the *ltaS/sgtB* suppressor strain S4-*sgtB* after growth in TSB sucrose medium
309 showed that, while the amount of crosslinked peptidoglycan was reduced in both strains
310 compared to the WT, the peptidoglycan in the suppressor strain was more crosslinked as
311 compared to the original *ltaS* mutant strain (Fig 7A and 7B). Taken together, our data highlight
312 that deletion of *ltaS* leads to a significant reduction in the amount of crosslinked peptidoglycan
313 in *S. aureus*. Inactivation of SgtB in a WT strain does not significantly affect the amount of
314 crosslinked peptidoglycan, as one might have expected based on the observed increase in
315 resistance oxacillin. However, in an LTA negative background strain, inactivation of SgtB
316 leads to an increase in peptidoglycan crosslinking, which might explain why an LTA negative
317 strain can grow in the absence of this monofunctional peptidoglycan glycosyltransferase.
318

319 **Discussion**

320 The anionic LTA polymer is a core component of the cell wall, essential for survival
321 and a number of studies have shown its importance in various cell processes (see review (22)).
322 Phenotypes caused by the depletion of LTA in *S. aureus* are misplacement and incomplete
323 formation of cell division septa, enlargement of the cells, together ultimately leading to cell

324 lysis (29, 34). However, how LTA mediates these roles is still unknown. In previous work, it
325 has been shown that LtaS and other core LTA synthesis proteins physically interact with early
326 and late stage cell division proteins as well as with a number of peptidoglycan synthesis
327 proteins (49). This indicates that LTA synthesis enzymes might at least transiently be part of
328 multi-enzyme complexes which might help to coordinate LTA synthesis with peptidoglycan
329 synthesis and cell division (49).

330 We have previously shown that *S. aureus* mutants producing increased intracellular c-
331 di-AMP levels can survive without LTA (34). It is now believed that at high c-di-AMP levels
332 and through the c-di-AMP-dependent regulation of potassium and osmolyte transporters, the
333 internal turgor pressure in the cell might be reduced so that the compromised LTA-depleted
334 cell wall can sustain the internal pressure (34, 50-52). Indeed, as part of the current study we
335 show that the absence of LTA leads to a sizable reduction in the amount of cross-linked
336 peptidoglycan in *S. aureus* (Fig. 7). This is consistent with the idea that in the absence of LTA
337 the cell wall is likely less able to sustain the high internal turgor pressure, which might also
338 contribute to the increased β-lactam sensitivity of LTA-negative strains observed in this and
339 previous studies (Table 2) (2, 34). The reason for the reduced amount of peptidoglycan
340 crosslinking is currently not clear but could potentially be due to mislocalization of PBPs in
341 the absence of LTA or PBPs having reduced enzyme activity since the proper ion homeostasis
342 cannot be maintained within the cell wall in the absence of LTA.

343 The aim of this study was to further elucidate the role of LTA in cell wall assembly and
344 potentially uncover additional proteins involved in the maintenance of cell wall integrity. A
345 suppressor screen followed by whole genome sequencing revealed mutations in genes coding
346 for ClpX, SgtB, MazE and VraT (Table 1) that can bypass the essentiality of LTA. Further
347 experimentation indicated that the strain with the mutation in *vraT* might not be a bona fide
348 suppressor strain. On the other hand, complementation analysis and growth assays on agar

349 plates containing the azo dye Congo red that prevents the growth of *S. aureus* by inhibiting the
350 LtaS enzyme (44) confirmed that inactivating mutations in *sgtB* and *mazE* could bypass the
351 essentiality of LTA. In a previous study it was found that an *S. aureus clpX* mutant readily
352 acquires inactivating mutations in *ltaS* resulting in the generation of LTA negative strains (2).
353 Conversely and consistent with this previous work, in this study we found in one of our LTA-
354 negative suppressor strains a large deletion in *clpX* (Table 1). ClpX is a protein folding
355 chaperon, which recognizes and targets proteins for degradation to the ClpP protease
356 component. In the absence of ClpX, *S. aureus* cells become smaller, show increased production
357 of autolysins and bacteria have a severe growth defect at temperatures of 30˚C or lower (2).
358 The introduction of loss of function mutations in the gene coding for the LTA synthase LtaS
359 in a *clpX* mutant alleviates some of these effects. This is perhaps due to LTA depletion having
360 the opposite effect that leads to an increase in cell size and decreased in autolysis as reported
361 in some previous publications (2, 31).

362 Mutations in the monofunctional glycosyltransferase SgtB were the most prevalent
363 mutations that arose in our suppressor screen. Previous studies have shown that SgtB is not
364 essential for the survival of the cell and as reported in this and a previous study, an *sgtB* mutant
365 strain does not show any obvious growth or morphological differences as compared to a WT
366 strain under standard growth conditions (Fig. 6) (15). Interestingly, we found that the *sgtB*
367 mutant strain LAC**sgtB::tn* was 4-fold more resistant to the cell-wall targeting antibiotic
368 oxacillin and this phenotype could be complemented by introduction of a wild-type copy of
369 *sgtB* (Table 2). These results are in accordance with previous reports, where strains with
370 mutations in genes that compensate for the lack of LTA, such as *gdpP* and *clpX* mutant strains,
371 also show increased oxacillin resistance (2, 34).

372 The increased resistance to cell wall-targeting antibiotics of the *sgtB* mutant prompted
373 us to investigate the peptidoglycan structure of an *sgtB* mutant in more detail, as we

374 hypothesized that in its absence changes such as an increased crosslinking might be observed
375 that could potentially explain the increased resistance. However, we could not detect any
376 obvious changes in the muropeptide profile of an *sgtB* mutant strain as compared to a WT strain
377 (Fig. 7). On the other hand, the original *ltaS* mutant strain showed a significant reduction of
378 around 30% in the higher oligomeric crosslinked peptidoglycan (Fig. 7). But perhaps most
379 importantly, the peptidoglycan isolated from the *sgtB/ltaS* double mutant S4-*sgtB* suppressor
380 strain showed an increase in peptidoglycan crosslinking as compared to the original *ltaS* mutant
381 strain (Fig. 7). We speculate that this increase in peptidoglycan cross-linking could potentially
382 strengthen the cell wall to better sustain the high internal turgor pressure and be at least in part
383 responsible for the observed growth improvement.

384 Bacterial two-hybrid studies have indicated that the *S. aureus* SgtB protein interacts
385 with SgtA, PBP1, PBP2 and PBP2A (15). Therefore, SgtB and the main glycosyltransferase
386 PBP2 might compete for substrate during peptidoglycan biosynthesis and inactivation of the
387 former might increase the substrate availability and activity of the latter. This might in turn aid
388 in the delivery of substrate to the transpeptidase domain of the bifunctional PBP2 enzyme
389 resulting in increased crosslinking. Alternatively, SgtB could also affect the function of PBP4,
390 which has been shown to be responsible for hyper-crosslinking of the staphylococcal
391 peptidoglycan (53). Clearly, more experiments are needed to clarify the complex nature of
392 peptidoglycan biosynthesis in *S. aureus* and changes observed in the absence of LTA.

393 Mutations in MazE could potentially also be tied in with changes in the cell wall
394 structure of *S. aureus*. The MazEF type II toxin-anitoxin system is part of the *sigB* regulon and
395 has been shown to be required for full activity of the alternative sigma factor SigB (37). It has
396 also been reported that overexpression of σ^B causes cell wall thickening in *S. aureus* and
397 increased resistance to cell wall-targeting antibiotics (38). Therefore, inactivation of MazE, as

398 observed in one of our suppressor strains, could potentially also affect cell wall homeostasis
399 via its effect on SigB.
400 In summary, our results suggest that in the absence of LTA, peptidoglycan in *S. aureus*
401 becomes less cross-linked, which might weaken the cell wall and become less able to sustain
402 the high internal turgor pressure, ultimately leading to cell lysis. The suppressor mutations
403 obtained in *sgtB* (and perhaps also in some of the other genes observed in our screen) help the
404 cell survive this detrimental effect by altering and strengthening the cell wall, likely allowing
405 the cell wall to again better withstand the high internal turgor pressure.
406
407 **Materials and Methods**
408 **Bacterial strains and culture conditions**. All bacterial strains used in this study are listed in
409 Table 3. *Escherichia coli* strains were cultured in Luria Bertani (LB) medium and
410 *Staphylococcus aureus* strains in tryptic soya broth (TSB) at 37°C with aeration, unless
411 otherwise stated. When appropriate the growth medium was supplemented with antibiotics and
412 inducers as follow: for *E. coli* cultures: ampicillin (Amp) 100 µg/ml, chloramphenicol (Cam)
413 10 µg/ml, and kanamycin (Kan) 30 µg/ml; for *S. aureus* cultures: chloramphenicol (Cam) 10
414 µg/ml for plasmid selection and 7.5 µg/ml for chromosomally integrated plasmid selection,
415 erythromycin (Erm) 10 µg/ml and kanamycin (Kan) 90 µg/ml. The inducer anhydrotetracycline
416 (Atet) was used at a concentration of 100 or 200 ng/ml in agar plates and 50 ng/ml in broth.
417
418 **Plasmid and strain construction**. Strains and primers used in this study are listed in Tables 3
419 and 4, respectively. Strain LAC**sgtB::tn* was generated by transduction using phage Φ85 and
420 transducing the *sgtB* region containing a transposon insertion in *sgtB* from the Nebraska
421 transposon mutant library strain NE596 (45) into strain LAC*. Plasmids piTET-*sgtB* and
422 piTET-*mazE* for anhydrotetracycline (Atet) inducible expression of *sgtB* and *mazE* in *S. aureus*

423 were generated by amplifying *sgtB* or *mazE* from LAC* chromosomal DNA using primers pair
424 ANG2268/ANG2269 or ANG2743/ANG2744, respectively. The PCR products and plasmid
425 piTET were digested with AvrII and SacII, ligated and then transformed into *E. coli* XL1-Blue
426 yielding strain XL1-Blue piTET-*sgtB* (ANG3926) and XL1-Blue piTET-*mazE* (ANG4727).
427 The plasmids were shuttled through *E. coli* strain IM08B (ANG3927 and ANG4728) and
428 subsequently electroporated into LAC* yielding strains LAC* piTET-*sgtB* (ANG4056) and
429 LAC* piTET-*mazE* (ANG4729) or into the respective suppressor strains yielding strains S4-
430 *sgtB* piTET-*sgtB* (ANG4058) and S2-*mazE* piTET-*mazE* (ANG4730). The plasmid pCL55-
431 *sgtB* for complementation and expression of *sgtB* from its native promoter was generated by
432 amplifying the *sgtB* gene including its native promoter region from LAC* chromosomal DNA
433 using primers ANG2270 and ANG2271. The PCR product and plasmid pCL55 were digested
434 with EcoRI and BamHI, ligated and then transformed into *E. coli* XL1-Blue yielding strain
435 XL1-Blue pCL55-*sgtB* (ANG4708), shuttled through IM08B (ANG3923) and subsequently
436 electroporated into the *sgtB* mutant strain yielding strain LAC**sgtB::tn* pCL55-*sgtB*
437 (ANG4711). WT LAC* or mutant *S. aureus* strains with the empty vectors piTET or pCL55
438 (see Table 3) were used as control strains in several experiments. The sequences of all plasmid
439 inserts were verified by automated fluorescence sequencing at GATC Biotechnology.
440
441 **Bacterial growth curves**. *S. aureus* LAC* and the indicated *ltaS* suppressor strains were
442 grown overnight in TSB medium containing the relevant antibiotic. The original *ltaS* mutant
443 strains were grown in TSB containing either 7.5% NaCl or 40% sucrose. Overnight cultures
444 were washed three times in TSB and diluted to a starting OD_{600} of 0.05. Cultures were
445 incubated at 37°C with aeration and OD_{600} values determined at 2 h intervals. WT LAC* and
446 the *sgtB* mutant strain LAC**sgtB::tn* were grown overnight in TSB or TSB supplemented with
447 10 µg/ml Erm. The next day, bacteria were washed three times in TSB and diluted to a starting

448 OD_{600} of 0.05 and 200 µl placed into a 96-well microtiter plate. Growth was monitored for 10h
449 using a SPECTROSTARnano plate reader (BMG Labtech). All growth curves were performed
450 in triplicates with average values and standard deviations plotted.
451
452 **Fluorescence microscopy analysis**. Cells from 1 ml overnight culture were collected by
453 centrifugation, washed 3 times in PBS pH 7.4 and 150 µl of these cell suspensions applied to
454 polylysine (0.1%, w/v) treated coverslips. Bacteria-coated coverslips were incubated with 100
455 µl of a 1 µg/ml BODIPY-vancomycin (Molecular Probes) solution in H_2O for 20 min, washed
456 and mounted on glass slides containing 20 µl Vectashield (Vector Laboratories). Slides were
457 viewed under a Zeiss Axio Imager A2, using a GFP filter set and images were captured with
458 an AxioCam MRc Rev.3 camera and analyzed using the Zen Pro 2012 SP2 software. The
459 experiment was performed in triplicate.
460
461 **Minimum Inhibitory Concentrations (MIC) determination**. Overnight cultures of WT, the
462 *sgtB* mutant and complementation stain as well as the indicated suppressor strains were grown
463 over night in TSB medium. The next day, cultures were adjusted to an OD_{600} of 0.05 in TSB
464 and 100 µl of these suspensions were incubated in 96-well plates with 2-fold dilutions of
465 various antimicrobials at the following starting concentrations: oxacillin 500 µg/ml or 1 µg/ml
466 as appropriate, daptomycin 32 µg/ml, lysostaphin 2 µg/ml, vancomycin 32 µg/ml and nisin 64
467 µg/ml. Oxacillin and daptomycin containing wells were supplemented with 2% (w/v) NaCl and
468 0.23 mM $CaCl_2$, respectively. Plates were incubated at 37°C for 24 hours with shaking at 500
469 rpm. MICs were determined as the concentration of antibiotic at which growth was inhibited
470 by >90% compared to growth without the antibiotic.
471

472 **LTA detection by western blot**. LTA extraction and detection by western blot was performed
473 as previously described (54). Briefly, samples were prepared from 1 ml overnight cultures
474 normalized based on OD_{600} readings, that is cells from 1 ml culture with an OD_{600} of 6 were
475 suspended in 90 µl 2 × SDS protein sample buffer. 10 µl of these samples were separated on
476 15% SDS-polyacrylamide gels and the material subsequently transferred to PVDF membranes.
477 LTA was detected using the monoclonal polyglycerolphosphate-specific LTA antibody
478 (Hycult Biotechnology) at a 1:4,000 dilution and the HRP-conjugated anti-mouse IgG antibody
479 (Cell Signaling Technologies, USA) at a 1:10,000 dilution. The blots were developed by
480 enhanced chemiluminescence using ClarityTM Western ECL Blotting Substrate (Bio-Rad) and
481 imaged using the ChemiDoc Touch Imaging System (Bio-Rad). Western blots were performed
482 in triplicates and a representative result is shown.
483
484 **Detection of WTA by Alcian blue silver staining.** Flasks with 60 ml TSB medium
485 supplemented with the appropriate antibiotics were inoculated with single colonies of WT
486 LAC* and different suppressor strains and the cultures incubated overnight (18 h) with shaking
487 at 37°C. The original *ltaS* mutant was grown in TSB supplemented with 40% sucrose and
488 incubated 6-8 hours longer. Cells from an OD_{600} equivalent of 120 were harvested by
489 centrifuging and the bacterial pellet stored at -20°C for further processing. Wall teichoic acid
490 extraction and detection by Alcian blue silver staining following SDS PAGE analysis was
491 performed as described in Covas *et al.* (55). Briefly, cells were washed in 20 ml buffer 1 (50
492 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.5), resuspended in 20 ml buffer 2
493 (buffer 1 with 4% (w/v) SDS) and boiled for 60 min. Next, the cells were washed with 20 ml
494 buffer 2 and after transfer to 2.0 ml reaction tubes washed once more with 1.6 ml buffer 2, 1.6
495 ml buffer 3 (buffer 1 with 2% (w/v) NaCl) and finally with 1.6 ml buffer 1. The samples were
496 suspended in 1.6 ml buffer 4 (20 mM Tris–HCl, pH 8.0, 0.5 % (w/v) SDS) and incubated for

497 4 h at 50°C with shaking following the addition of 2 µl of Proteinase K solution (20 mg /ml)
498 from *Tritirachium album*. Next, the cells were collected by centrifugation, washed once with
499 1.6 ml buffer 3 and three times with 1.6 ml of H_2O. To release the WTA, the pellets were
500 suspended in 1 ml of 0.1 mM NaOH and the samples incubated for 16 h at 25°C. Next, the
501 samples were centrifuged and 30 µl of the supernatant containing WTA separated on native
502 Tris/Tricine polyacrylamide gels and the WTA visualized by Alcian blue silver staining (55).
503
504 **Whole genome sequencing**. WT LAC*, LAC*Δ$ltaS_N$, LAC*Δ$ltaS_S$, and the indicated
505 suppressor strains were cultured overnight at 37°C, cells harvested and genomic DNA
506 extracted. Genome sequencing was performed by MicrobesNG (University of Birmingham)
507 using an Illumina platform and a 250 bp paired end read kit. Sequence analysis was performed
508 using the CLC Genomics work bench software package. First the LAC* reads were aligned
509 against the published USA300 FPR3757 genome sequence (RefSeq accession number
510 NC_007793.1), assembled into a reference contig and the USA300 FPR3757 annotation
511 transferred onto the LAC* sequence. Next, the Illumina reads for the original *ltaS* mutant
512 strains LAC*Δ$ltaS_S$ and LAC*Δ$ltaS_N$ as well as the seven different suppressor strains were
513 mapped onto the assembled LAC* sequence and high frequency (> 65%) and good quality base
514 changes identified using the CLC Genomics Workbench software package. Genomic
515 alternations found in the suppressor strains but not present in the original *ltaS* mutant strain are
516 summarized in Table 1. The Illumina short read for the WT LAC* has been previously
517 published (35) and has been deposited in the European Nucleotide Archive under the study
518 accession number PRJEB14759. The Illumina reads for the *ltaS* mutants and *ltaS* suppressor
519 strains were deposited in the European Nucleotide Archive under the study accession number
520 PRJEB14723.
521

522 **Peptidoglycan isolation and analysis.** Overnight cultures of *S. aureus* strains LAC*, S4-*sgtB*
523 and LAC**sgtB::tn* were prepared in TSB or TSB supplemented with 40% sucrose and of the
524 original *ltaS* mutant strain LAC*Δ$ltaS_N$ (ANG2134) in TSB supplemented with 40% sucrose.
525 The next day, cells were back-diluted in 2 litres of the same growth medium to an OD_{600} of
526 0.05. The cultures were grown at 37°C until they reached an OD_{600} of approximately 1.5,
527 cooled on ice and cells were collected by centrifugation. Peptidoglycan was purified and
528 digested with mutanolysin as previously described (34, 48). HPLC analysis of the digested
529 peptidoglycan material was performed as described previously (48) and the muropeptide
530 profiles were determined for each strain and growth condition in triplicates. For the
531 quantification of monomeric, dimeric, trimeric and higher oligomeric peptidoglycan material,
532 the peaks were integrated. The total peak area for each muropetide profile was determined and
533 set to 100% and the % monomeric, dimeric, trimeric, and higher oligomeric peaks calculated
534 and the average values and standard deviation from the three profiles determined and plotted.
535
536 **c-di-AMP quantification by competitive ELISA.** Five ml TSB were inoculated with a single
537 colony of *S. aureus* LAC* (WT), strain LAC* *gdpP::kan,* US3 (LAC*Δ$ltaS_S$*::erm* suppressor
538 with mutation in *gdpP*) as well as the different LTA-negative suppressor and the tubes were
539 incubated for 8 hours at 37°C. Next, the cultures were back-diluted to an OD_{600} of 0.05 in 10
540 ml TSB and grown for 15 hours at 37°C. Bacterial cells from these cultures were collected by
541 centrifugation, cell lysates prepared and the cellular c-di-AMP levels determined using a
542 previously described competitive ELISA method (35, 36). However, in place of determining
543 c-di-AMP levels based on a standard curve the A_{490} values were directly compared. To this
544 end, the A_{490} reading obtained for samples derived for the WT strain was set to 100% and the
545 % values calculated for the samples derived from the other strains. Thee independent
546 experiments were performed with three technical replicates and the average values and

547 standard deviation of the % A_{490} values determined for each strain as compared to the WT
548 strain. Of note, since this is a competitive ELISA, and a decrease in A_{490} readings represents
549 an increase in cellular c-di-AMP levels.
550

551 **Acknowledgments**

552 This research was supported by the European Research Council grant 260371 and the
553 Wellcome Trust grant 100289 to AG, the UK Medical Research Council (MRC) within the
554 AMR Cross-council initiative Collaborative Grant MR/N002679/1 to WV and the German
555 Research Foundation [Deutsche Forschungsgemeinschaft (DFG)] grant SCHU 3159/1-1 to
556 CFS. The Illumina sequencing was performed by MicrobesNG, which was supported by the
557 BBSRC grant BB/L024209/1.

558 **Table 1: Genomic variations detected in the suppressor strains as compared the original**
559 ***ltaS* mutant strains**
560

Strain[1]	Ref. pos.[2]	Type[3]	Ref.[4]	Allele[5]	Freq.[6]	Av. Qual.[7]	Annotations	Amino acid change[8]
ANG3711 S1-*sgtB*	2018191 2017952-2018187	SVN DEL	T	A	100	38.5	SAUSA300_1855, monofunctional glycosyltransferase SgtB SAUSA300_1855, monofunctional glycosyltransferase SgtB	Asn11Tyr
ANG3712 S2-*mazE*	1380637 1380639 1380641 2188437-2188446	INS SNV REP DEL	- C C TATCGGAAAA	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*	1775491 1775495 1775668 1775500-1775663	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*	2017704	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
ANG3717 S7-*vraT*	2027736	SNV	A	C	100	38.8	SAUSA300_1867, membrane protein Yvfq/VraT	Leu71*
ANG3718 S8-*sgtB*	2018107	INS	-	T	92.6	38.6	SAUSA300_1855, monofunctional glycosyltransferase SgtB	Ser39fs

561 [1]Suppressors S1, S2, S7 and S8 were derived from strain LAC*Δ$ltaS_S$ (ANG2135) and
562 suppressor strains S3, S4 and S6 were derived from strain LAC*Δ$ltaS_N$ (ANG2134).
563 [2]Reference position indicates base number in the LAC* reference genome
564 (USA300_FPR3757, NCBI CP000255.1). [3]Type of mutation with DEL indicating deletion,
565 SNV indicating single nucleotide variation, INS indicating insertion and REP indicating
566 replacement. [4]Ref. indicates base in the LAC* reference genome. [5]Allele indicates base in the
567 sequenced strain. [6]Freq. indicates frequency of the specific variant found in the sample reads.

568 [7]Av. Qual. denotes the average base quality score at the indicated position. [8]Amino acid change

569 in the encoded protein in LAC* versus the sequenced strain with fs indicating a frame shift and

570 * a premature stop codon.

571

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

	lysostaphin	nisin	vancomycin	daptomycin
LAC*	**0.125** (0.125, 0.125, 0.125)	**16** (16, 16, 16)	**4** (4, 4, 4)	**16** (16, 16, 16)
S2-*mazE*	**0.125** (0.125, 0.125, 0.125)	**2-4** (2, 2, 4)	**2** (2, 2, 2)	**4-8** (8, 8, 4)
S4-*sgtB*	**0.125-0.250** (0.125, 0.125, 0.25)	**4** (4, 4, 4)	**1** (1, 1, 1)	**8-16** (16, 8, 8)
S7-*vraT*	**0.500** (0.5, 0.5, 0.5)	**2-4** (2, 2, 4)	**4-8** (8, 4, 4)	**8** (8, 8, 8)
sgtB::tn	**0.125-0.250** (0.25, 0.125, 0.125)	**16-32** (32, 16, 32)	**4** (4, 4, 4)	**16** (16, 16, 16)
sgtB::tn **pCL55**	**0.125-0.250** (0.125, 0.125, 0.25)	**8-16** (16, 8, 16)	**4** (4, 4, 4)	**16** (16, 16, 16)
sgtB::tn **pCL55-*sgtB***	**0.125** (0.125, 0.125, 0.125)	**8-16** (8, 16, 16)	**4-8** (4, 8, 4)	**16** (16, 16, 16)

573

574 The MIC was defined as antibiotic concentration that leads to >90% growth inhibition as
575 compared to growth without antibiotic. MICs were determined in triplicate. The obtained MIC
576 range is shown in bold and individual MIC values of the replicate samples are shown below in
577 parenthesis.

578

579 **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	(56)
ANG3732	IM08B pCL55; AmpR	(52)
ANG3926	XL1-Blue piTET-*sgtB*; AmpR	This study
ANG3927	IM08B piTET-*sgtB*; AmpR	This study
ANG3928	IM08B piTET; AmpR	(57)
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	(58)
NE596	JE2*sgtB::tn*; NARSA library transposon mutant; ErmR – ANG3930	(45)
ANG1961	LAC**gdpP::kan,* KanR	(34)
ANG2134	LAC*Δ*ltaS*$_N$*::erm* pCN38; Isolated on 7.5% NaCl; CamR ErmR	(34)
ANG2135	LAC*Δ*ltaS*$_S$*::erm* pCN38; Isolated on 40% Sucrose; CamR ErmR	(34)
ANG2137	LAC*Δ*ltaS*$_N$*::erm* suppressor S6 original colony; ErmR	(34)
ANG2140	LAC*Δ*ltaS*$_S$*::erm* suppressor S7 original colony; ErmR	(34)
ANG2143	LAC*Δ*ltaS*$_S$*::erm* suppressor S8 original colony; ErmR	(34)
ANG2434	LAC*Δ*ltaS*$_S$*::erm* suppressor with *gdpP* mutation (pass 4) (short: US3); ErmR	(34)
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	(57)
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

580

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

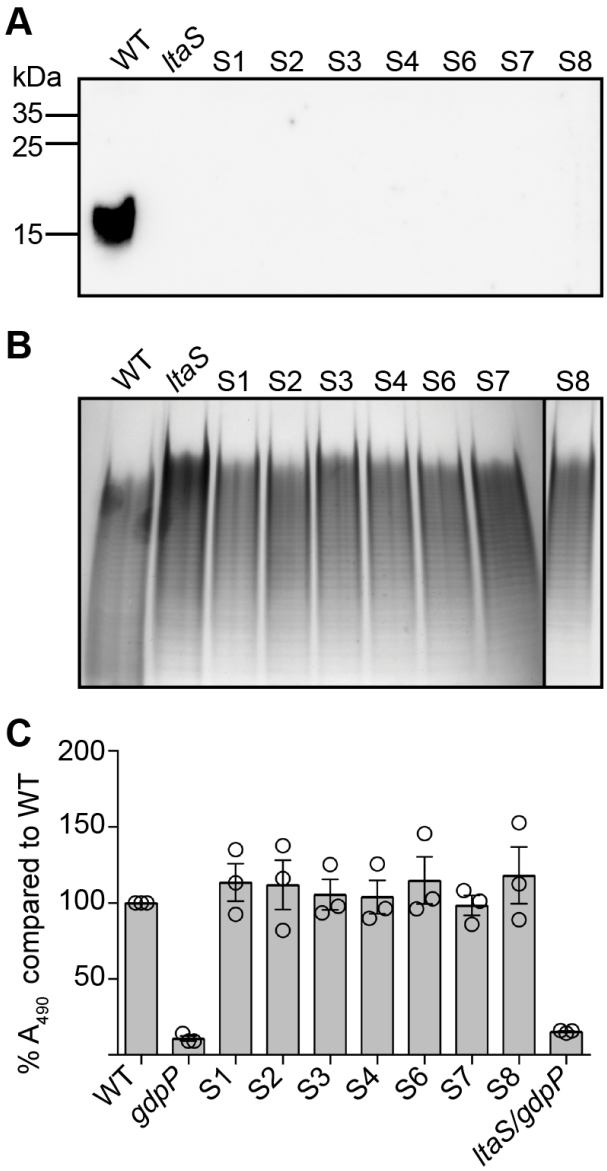
Primer ID	Name	Sequence
ANG2229	sausa300_1867 Fw seq	GAAGCATTTCAAATTCAATGTGCAAG
ANG2230	sausa300_1867 Rv seq	GAAGCATTTCAAATTCAATGTGCAAG
ANG2231	sgtB Fw seq	GTCGTCTCTATTGGCATTTAATAGGG
ANG2232	sgtB Rv seq	AATAATGAAGTACTAATCAAGTGGC
ANG2233	sausa300_1254 Fw seq	CATTTTGAAGAAAGGATTAATCAA
ANG2234	sausa300_1254 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

582 Restriction site sequences are underlined.

583
584

585 **Figures and Legends**

586



587
588
589

590 **Figure 1: Detection of LTA by western blot, WTA by Alcian blue silver staining and c-**

591 **di-AMP using a competitive ELISA method.** (A) Detection of LTA by western-blot. Cell

592 extracts were prepared from LAC* (WT), an *ltaS* mutant (strain ANG2135) and the 7 *ltaS*

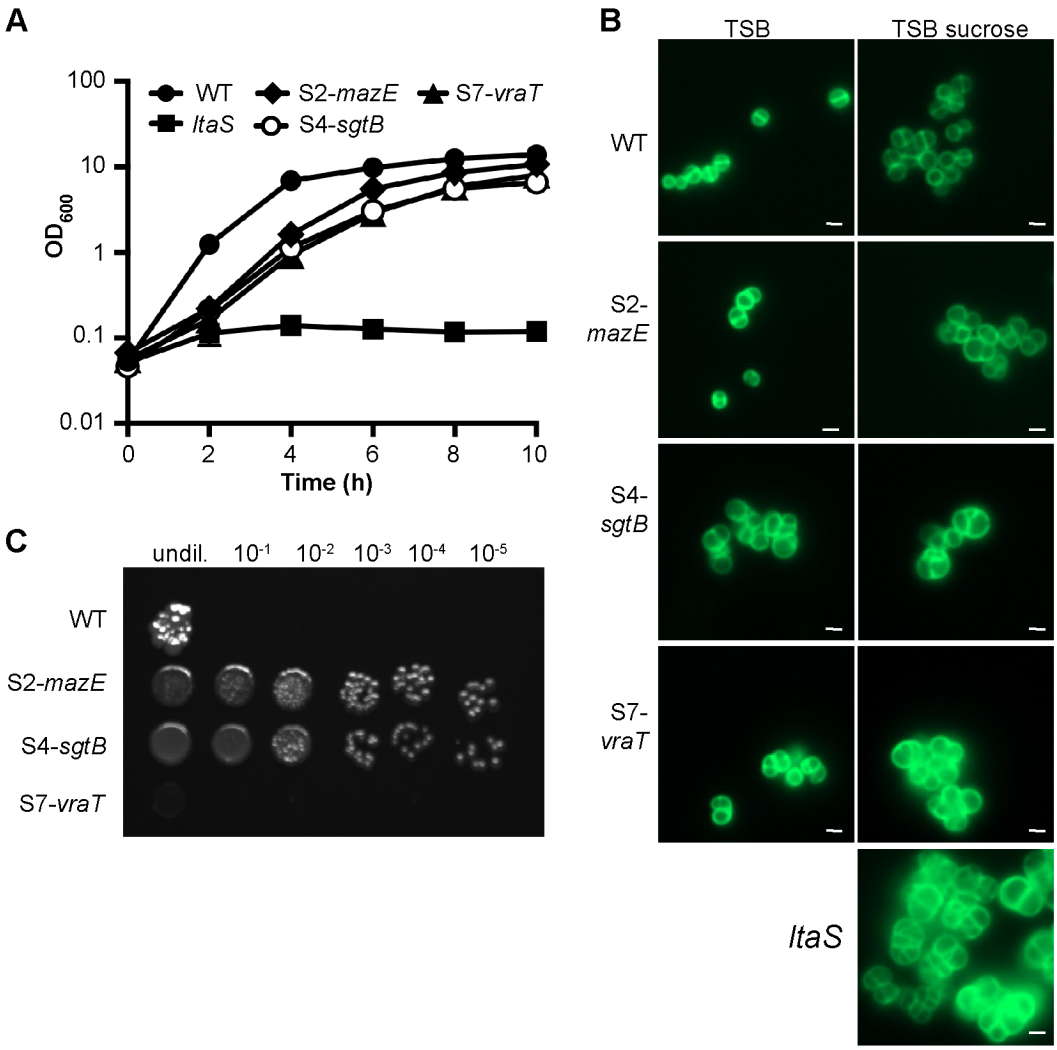
593 suppressor strains (S1-S4 and S6-S8) and separated on a 15% PAA gel. LTA was subsequently

594 detected by western blot using a monoclonal polyglycerolphosphate specific antibody. A

595 representative result from 3 independent experiments is shown. (B) Visualization of wall

596 teichoic acid (WTA) by native PAGE. WTAs were isolated from the same strains as shown in

597 (A) and separated on a 20% native gel. Bands were visualized by Alcian blue and subsequently
598 silver stain. One representative result from 4 independent experiments is shown. (C) c-di-AMP
599 detection by competitive ELISA. Cytoplasmic extracts were prepared from LAC* (WT), the
600 high c-di-AMP level control strains LAC* *gdpP::kan* (*gdpP*) and US3 (*ltaS/gdpP*) as well as
601 from the 7 *ltaS* suppressor strains (S1-S4 and S6-S8). c-di-AMP amounts were determined and
602 compared to the WT strain by ELISA. Of note, as this is a competitive ELISA assay, lower
603 A_{490} readings are obtained for samples with higher c-di-AMP levels. The A_{490} reading obtained
604 for the sample derived from the WT strain was set to 100% and % A_{490} reading calculated for
605 the test strains. The average % values and standard deviations from three independent
606 experiments (with three technical replicates) are plotted.

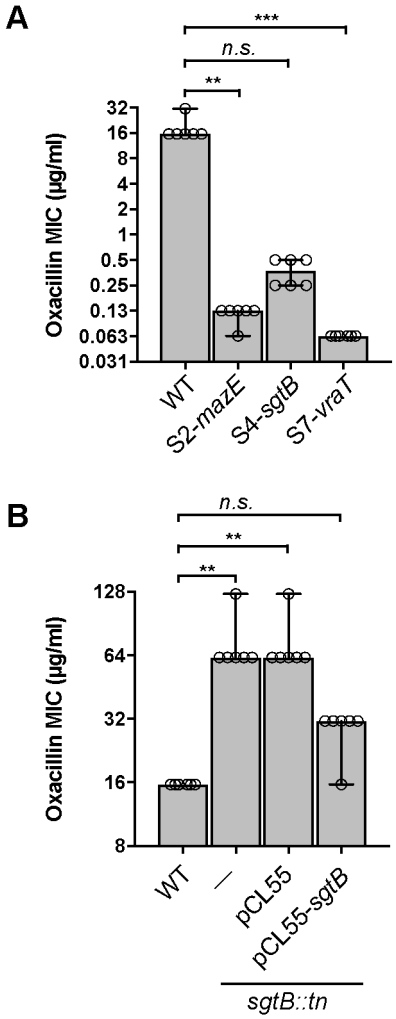


607
608

609 **Figure 2: Growth and cell morphology of WT and mutant *S. aureus* strains.** (A) Bacterial
610 growth curves. LAC* (WT) and the suppressor strains S2-*mazE,* S4-*sgtB* and S7-*vraT* were
611 grown overnight in TSB and the original *ltaS* mutant strain (ANG2135) in TSB with 40%
612 sucrose. Next day, bacterial cells were washed and diluted in TSB to an OD_{600} of 0.05 and the
613 bacterial growth was subsequently monitored over a period of 10 hours. The average OD_{600}
614 readings from three experiments were plotted. (B) Microscopic analysis. Bacterial cells from
615 overnight cultures of the same strains as used in panel A were washed, back-diluted and grown
616 to mid-log phase in TSB or TSB with 40% sucrose as indicated. The bacterial cells were
617 subsequently stained with BODIPY-vancomycin and viewed by fluorescent microscopy. (C)
618 Analysis of bacterial growth on Congo red-containing TSA plates. Overnight cultures of WT

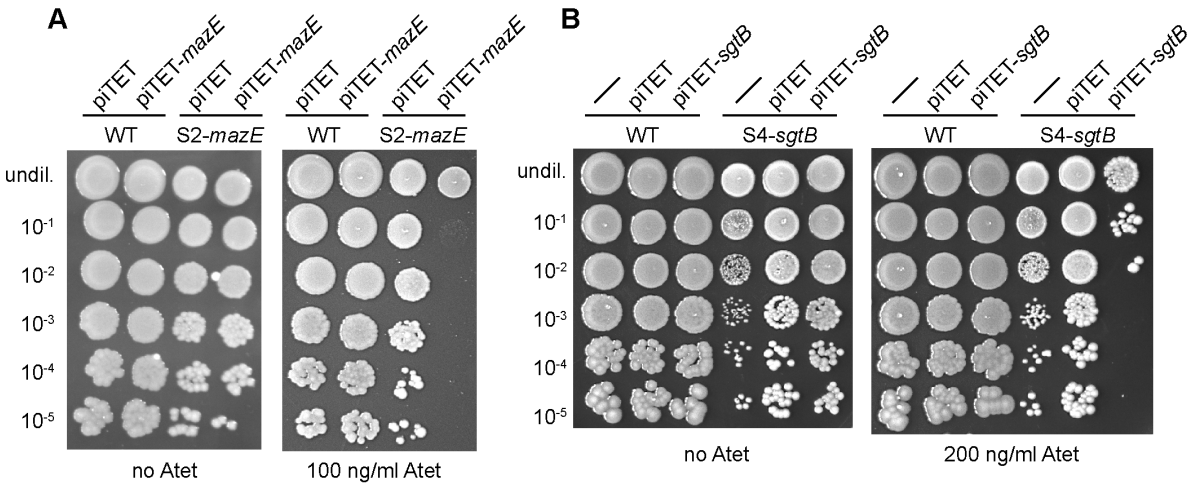
619 and the indicated suppressor strains were serially diluted and spotted on TSA plates
620 supplemented with 0.1% Congo red and the plates incubated for 48h at 37˚C. For B and C, a
621 representative result of three independent experiments is shown.
622

623



624

625 **Figure 3: Oxacillin MICs for WT and mutant *S. aureus* strains.** Oxacillin minimal
626 inhibitory concentrations (MICs) of (A) LAC* (WT) and the suppressor strains S2-*mazE,* S4-
627 *sgtB* and S7-*vraT* and (B) LAC* (WT), the LAC* *sgtB::tn* mutant either without integrative
628 plasmid (-), with integrative plasmid (pCL55) or integrative complementation plasmid
629 (pCL55-*sgtB*). Experiments were conducted with six biological replicates. Medians and 95%
630 confidence intervals for all strains are plotted. A Kruskal-Wallis one-way analysis of variance
631 tests was performed, which indicated significant differences. Subsequently, Dunn's tests were
632 performed and the p-values corrected for the multiple comparisons against the WT. p-values <
633 0.01 (**) and < 0.001 (***).



634
635
636 **Figure 4: Growth complementation analysis.** TSA plates with spot dilutions. (A) *S. aureus*
637 strains LAC* piTET, LAC* piTET-*mazE*, S2-*mazE* piTET or S2-*mazE* piTET-*mazE* were
638 grown overnight in TSB supplemented with chloramphenicol, washed twice in PBS and serial
639 dilutions spotted on TSA plates supplemented with 7.5 ng/ml chloramphenicol without (left
640 panel) or with 100 ng/ml Atet (right panel). (B) *S. aureus* strains LAC* (WT), LAC* piTET,
641 LAC* piTET-sgtB, S4-*sgtB*, S4-*sgtB* piTET or S4-*sgtB* piTET-*sgtB* were grown and samples
642 prepared as described in A, however without chloramphenicol selection, and dilutions were
643 spotted on TSA plates (left panel) or TSA plates supplemented with 200 ng/ml Atet (right
644 panel). Representative plate images from three independent experiments are shown.
645
646

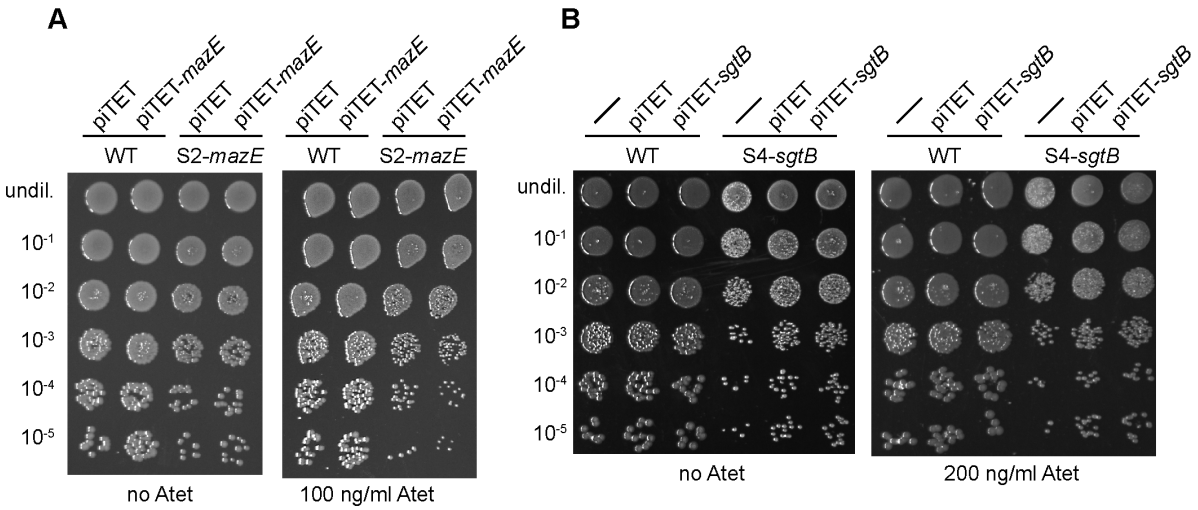
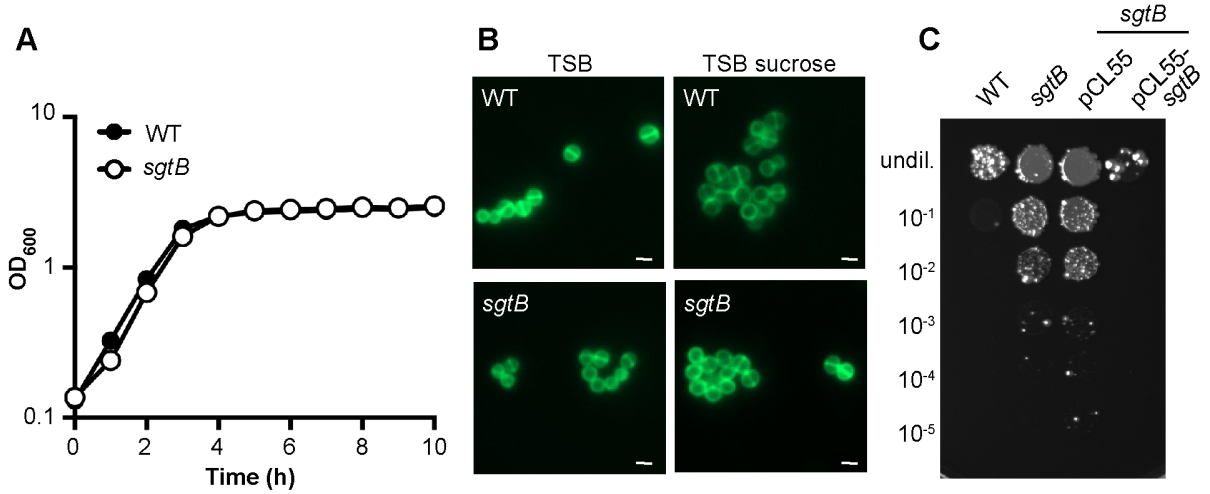


Figure 5: Growth complementation analysis. TSA sucrose plates with spot dilutions. (A) *S. aureus* strains LAC* piTET, LAC* piTET-*mazE,* S2-*mazE* piTET or S2-*mazE* piTET-*mazE* were grown overnight in TSB supplemented with chloramphenicol, washed twice in PBS and serial dilutions spotted on TSA 40% sucrose plates supplemented with 7.5 ng/ml chloramphenicol without (left panel) or with 100 ng/ml Atet (right panel). (B) *S. aureus* strains LAC* (WT), LAC* piTET, LAC* piTET-*sgtB,* S4-*sgtB,* S4-*sgtB* piTET or S4-*sgtB* piTET-*sgtB* were grown and samples prepared as described in A, however without chloramphenicol selection, and dilutions were spotted on TSA 40% sucrose plates (left panel) or TSA 40% sucrose plates supplemented with 200 ng/ml Atet (right panel). Representative plate images from three independent experiments are shown.

660



661
662

663 **Figure 6: Growth and cell morphology of *S. aureus* strain LAC*sgtB::tn.** (A) Bacterial
664 growth curves. Overnight cultures of LAC* (WT) and strain LAC*sgtB::tn (sgtB) were diluted
665 in TSB to an OD_{600} of 0.05 and the growth monitored over a period of 10 hours using a plate
666 reader. The average OD_{600} values and standard deviation from three independent experiments
667 are plotted. (B) Microscopy analysis. *S. aureus* strains LAC* (WT) and LAC*sgtB::tn (sgtB)
668 were grown overnight in TSB or TSB supplemented with 40% sucrose, stained with BODIPY-
669 vancomycin and viewed under a fluorescence microscope. (C) Susceptibility to Congo red.
670 Overnight cultures of strains LAC* (WT) and the *sgtB* mutant strains LAC*sgtB::tn,
671 LAC*sgtB::tn pCL55 as well as the complementation strain LAC*sgtB::tn pCL55-sgtB were
672 serially diluted and aliquots spotted on TSA plates supplemented with 0.1% Congo red. For
673 panels B and C, representative result from three independent experiments are shown.

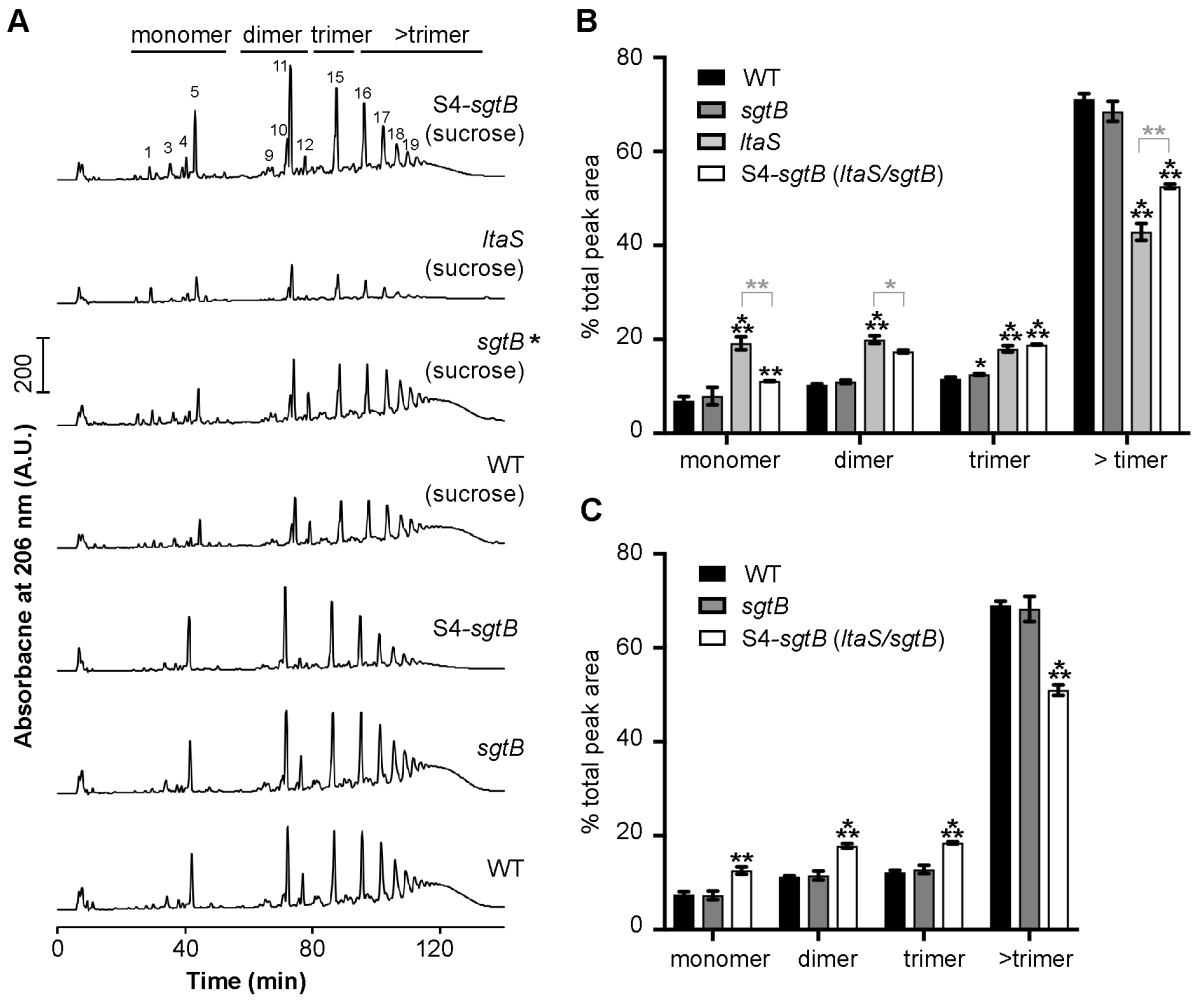


Figure 7: Peptidoglycan analysis of WT and mutant *S. aureus* strains. (A) HPLC profile of mutanolysin digested peptidoglycan. Peptidoglycan was isolated and digested with mutanolysin as described in the materials and methods section from *S. aureus* strains LAC* (WT), LAC**sgtB::tn* (*sgtB*) and the 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. The individual muropeptide peaks were labelled where possible with the numbers as described in de Jonge *et al*. (48). (B-C) Quantification of the different peptidoglycan peaks. The peaks corresponding to monomeric, dimeric, trimeric and > trimeric

687 peptidoglycan fragments were integrated and quantified for the strains used in panel A. The
688 combined peak area was set to 100% for each strain and the average values and standard
689 deviations from the three independent peptidoglycan isolations were plotted in panel B, for the
690 strains grown in TSB sucrose medium and in panel C for the strains grown in TSB medium.
691 Unpaired two-tailed student's T-tests were used to determine statistically significant
692 differences in monomer, dimer, trimer or > trimer fractions between WT and mutant strains.
693 The obtained p-values were multiplied by the Bonferroni correction factors 3 (panel B) and 2
694 (panel C) to consider that the WT peaks were compared to three or two different strains,
695 respectively. Statistically significant differences are indicated by black asterisks. For panel B,
696 unpaired two-tailed student's T-tests were also used to determine statistically significant
697 differences in monomer, dimer, trimer or > trimer fractions between the *ltaS* mutant and the
698 other three strains and the statistically significant differences between the *ltaS* mutant and S4-
699 *ltaS* suppressor are shown with grey asterisks. p-values < 0.05 (*), < 0.01 (**) and < 0.001
700 (***).
701

702 **REFERENCES:**

703

704 1. **Kluytmans J, Van Belkum A, Verbrugh H.** 1997. Nasal carriage of *Staphylococcus*
705 *aureus*: epidemiology, underlying mechanisms, and associated risks. Clinical
706 microbiology reviews **10:**505-520.

707 2. **Baek KT, Bowman L, Millership C, Dupont Sogaard M, Kaever V, Siljamaki P,**
708 **Savijoki K, Varmanen P, Nyman TA, Gründling A, Frees D.** 2016. The Cell Wall
709 Polymer Lipoteichoic Acid Becomes Nonessential in *Staphylococcus aureus* Cells
710 Lacking the ClpX Chaperone. MBio **7**.

711 3. **Francis JS, Doherty MC, Lopatin U, Johnston CP, Sinha G, Ross T, Cai M, Hansel**
712 **NN, Perl T, Ticehurst JR.** 2005. Severe community-onset pneumonia in healthy adults
713 caused by methicillin-resistant *Staphylococcus aureus* carrying the Panton-Valentine
714 leukocidin genes. Clinical Infectious Diseases **40:**100-107.

715 4. **Hiramatsu K, Katayama Y, Matsuo M, Sasaki T, Morimoto Y, Sekiguchi A, Baba**
716 **T.** 2014. Multi-drug-resistant *Staphylococcus aureus* and future chemotherapy. Journal
717 of Infection and Chemotherapy **20:**593-601.

718 5. **Horn J, Stelzner K, Rudel T, Fraunholz M.** 2017. Inside job: *Staphylococcus aureus*
719 host-pathogen interactions. International Journal of Medical Microbiology.

720 6. **Foster TJ, Geoghegan JA, Ganesh VK, Höök M.** 2014. Adhesion, invasion and
721 evasion: the many functions of the surface proteins of *Staphylococcus aureus*. Nat Rev
722 Microbiol **12:**49-62.

723 7. **Silhavy TJ, Kahne D, Walker S.** 2010. The bacterial cell envelope. Cold Spring
724 Harbor perspectives in biology **2:**a000414.

725 8. **Rajagopal M, Walker S.** 2015. Envelope structures of gram-positive bacteria, p 1-44,
726 Protein and Sugar Export and Assembly in Gram-positive Bacteria. Springer.

727 9. **Vollmer W, Blanot D, De Pedro MA.** 2008. Peptidoglycan structure and architecture.
728 FEMS microbiology reviews **32:**149-167.
729 10. **Archibald A, Hancock I, Harwood C.** 1993. Cell wall structure, synthesis, and
730 turnover, p 381-410, *Bacillus subtilis* and other Gram-positive bacteria. American
731 Society of Microbiology.
732 11. **Holtje JV.** 1998. Growth of the stress-bearing and shape-maintaining murein sacculus
733 of *Escherichia coli*. Microbiol Mol Biol Rev **62:**181-203.
734 12. **Reed P, Atilano ML, Alves R, Hoiczyk E, Sher X, Reichmann NT, Pereira PM,**
735 **Roemer T, Filipe SR, Pereira-Leal JB, Ligoxygakis P, Pinho MG.** 2015.
736 *Staphylococcus aureus* Survives with a Minimal Peptidoglycan Synthesis Machine but
737 Sacrifices Virulence and Antibiotic Resistance. PLoS Pathog **11:**e1004891.
738 13. **Hartman BJ, Tomasz A.** 1984. Low-affinity penicillin-binding protein associated
739 with beta-lactam resistance in *Staphylococcus aureus*. J Bacteriol **158:**513-516.
740 14. **Pinho MG, de Lencastre H, Tomasz A.** 2001. An acquired and a native penicillin-
741 binding protein cooperate in building the cell wall of drug-resistant staphylococci. Proc
742 Natl Acad Sci U S A **98:**10886-10891.
743 15. **Reed P, Veiga H, Jorge AM, Terrak M, Pinho MG.** 2011. Monofunctional
744 transglycosylases are not essential for *Staphylococcus aureus* cell wall synthesis. J
745 Bacteriol **193:**2549-2556.
746 16. **Wang QM, Peery RB, Johnson RB, Alborn WE, Yeh WK, Skatrud PL.** 2001.
747 Identification and characterization of a monofunctional glycosyltransferase from
748 *Staphylococcus aureus*. J Bacteriol **183:**4779-4785.
749 17. **Weidenmaier C, Peschel A.** 2008. Teichoic acids and related cell-wall glycopolymers
750 in Gram-positive physiology and host interactions. Nature Reviews Microbiology
751 **6:**276.

752 18. **Kawai Y, Marles-Wright J, Cleverley RM, Emmins R, Ishikawa S, Kuwano M,**
753 **Heinz N, Bui NK, Hoyland CN, Ogasawara N, Lewis RJ, Vollmer W, Daniel RA,**
754 **Errington J.** 2011. A widespread family of bacterial cell wall assembly proteins. Embo
755 j **30:**4931-4941.
756 19. **Kern T, Giffard M, Hediger S, Amoroso A, Giustini C, Bui NK, Joris B, Bougault**
757 **C, Vollmer W, Simorre JP.** 2010. Dynamics characterization of fully hydrated
758 bacterial cell walls by solid-state NMR: evidence for cooperative binding of metal ions.
759 J Am Chem Soc **132:**10911-10919.
760 20. **Neuhaus FC, Baddiley J.** 2003. A continuum of anionic charge: structures and
761 functions of D-alanyl-teichoic acids in gram-positive bacteria. Microbiology and
762 Molecular Biology Reviews **67:**686-723.
763 21. **Brown S, Santa Maria JP, Jr., Walker S.** 2013. Wall teichoic acids of gram-positive
764 bacteria. Annu Rev Microbiol **67:**313-336.
765 22. **Percy MG, Gründling A.** 2014. Lipoteichoic acid synthesis and function in gram-
766 positive bacteria. Annu Rev Microbiol **68:**81-100.
767 23. **Percy MG, Karinou E, Webb AJ, Gründling A.** 2016. Identification of a
768 Lipoteichoic Acid Glycosyltransferase Enzyme Reveals that GW-Domain-Containing
769 Proteins Can Be Retained in the Cell Wall of Listeria monocytogenes in the Absence
770 of Lipoteichoic Acid or Its Modifications. J Bacteriol **198:**2029-2042.
771 24. **Santa Maria JP, Jr., Sadaka A, Moussa SH, Brown S, Zhang YJ, Rubin EJ,**
772 **Gilmore MS, Walker S.** 2014. Compound-gene interaction mapping reveals distinct
773 roles for *Staphylococcus aureus* teichoic acids. Proc Natl Acad Sci U S A **111:**12510-
774 12515.

775 25. **Peschel A, Otto M, Jack RW, Kalbacher H, Jung G, Götz F.** 1999. Inactivation of
776 the dlt Operon in *Staphylococcus aureus* Confers Sensitivity to Defensins, Protegrins,
777 and Other Antimicrobial Peptides. Journal of Biological Chemistry **274:**8405-8410.
778 26. **Kho K, Meredith TC.** 2018. Salt-Induced Stress Stimulates a Lipoteichoic Acid-
779 Specific Three Component Glycosylation System in *Staphylococcus aureus*. J Bacteriol
780 doi:10.1128/JB.00017-18.
781 27. **Reichmann NT, Gründling A.** 2011. Location, synthesis and function of glycolipids
782 and polyglycerolphosphate lipoteichoic acid in Gram-positive bacteria of the phylum
783 Firmicutes. FEMS Microbiol Lett **319:**97-105.
784 28. **Lu D, Wörmann ME, Zhang X, Schneewind O, Gründling A, Freemont PS.** 2009.
785 Structure-based mechanism of lipoteichoic acid synthesis by *Staphylococcus aureus*
786 LtaS. Proceedings of the National Academy of Sciences **106:**1584-1589.
787 29. **Gründling A, Schneewind O.** 2007. Synthesis of glycerol phosphate lipoteichoic acid
788 in *Staphylococcus aureus*. Proc Natl Acad Sci U S A **104:**8478-8483.
789 30. **Karatsa-Dodgson M, Wormann ME, Gründling A.** 2010. In vitro analysis of the
790 *Staphylococcus aureus* lipoteichoic acid synthase enzyme using fluorescently labeled
791 lipids. J Bacteriol **192:**5341-5349.
792 31. **Oku Y, Kurokawa K, Matsuo M, Yamada S, Lee BL, Sekimizu K.** 2009. Pleiotropic
793 roles of polyglycerolphosphate synthase of lipoteichoic acid in growth of
794 *Staphylococcus aureus* cells. J Bacteriol **191:**141-151.
795 32. **Schirner K, Marles-Wright J, Lewis RJ, Errington J.** 2009. Distinct and essential
796 morphogenic functions for wall-and lipo-teichoic acids in *Bacillus subtilis*. The EMBO
797 journal **28:**830-842.
798 33. **Fedtke I, Mader D, Kohler T, Moll H, Nicholson G, Biswas R, Henseler K, Götz
799 F, Zähringer U, Peschel A.** 2007. A *Staphylococcus aureus ypfP* mutant with strongly

800 reduced lipoteichoic acid (LTA) content: LTA governs bacterial surface properties and
801 autolysin activity. Molecular microbiology **65:**1078-1091.
802 34. **Corrigan RM, Abbott JC, Burhenne H, Kaever V, Gründling A.** 2011. c-di-AMP
803 is a new second messenger in *Staphylococcus aureus* with a role in controlling cell size
804 and envelope stress. PLoS Pathog **7:**e1002217.
805 35. **Bowman L, Zeden MS, Schuster CF, Kaever V, Gründling A.** 2016. New Insights
806 into the Cyclic Di-adenosine Monophosphate (c-di-AMP) Degradation Pathway and
807 the Requirement of the Cyclic Dinucleotide for Acid Stress Resistance in
808 *Staphylococcus aureus*. J Biol Chem **291:**26970-26986.
809 36. **Underwood AJ, Zhang Y, Metzger DW, Bai G.** 2014. Detection of cyclic di-AMP
810 using a competitive ELISA with a unique pneumococcal cyclic di-AMP binding
811 protein. Journal of microbiological methods **107:**58-62.
812 37. **Donegan NP, Cheung AL.** 2009. Regulation of the mazEF toxin-antitoxin module in
813 *Staphylococcus aureus* and its impact on sigB expression. Journal of bacteriology
814 **191:**2795-2805.
815 38. **Morikawa K, Maruyama A, Inose Y, Higashide M, Hayashi H, Ohta T.** 2001.
816 Overexpression of sigma factor, sigma(B), urges *Staphylococcus aureus* to thicken the
817 cell wall and to resist beta-lactams. Biochem Biophys Res Commun **288:**385-389.
818 39. **Guldimann C, Boor KJ, Wiedmann M, Guariglia-Oropeza V.** 2016. Resilience in
819 the face of uncertainty: sigma factor B fine-tunes gene expression to support
820 homeostasis in gram-positive bacteria. Applied and environmental microbiology
821 **82:**4456-4469.
822 40. **Kuroda M, Kuroda H, Oshima T, Takeuchi F, Mori H, Hiramatsu K.** 2003. Two-
823 component system VraSR positively modulates the regulation of cell-wall biosynthesis
824 pathway in *Staphylococcus aureus*. Mol Microbiol **49:**807-821.

825 41. **Kuroda M, Kuwahara-Arai K, Hiramatsu K.** 2000. Identification of the up- and
826 down-regulated genes in vancomycin-resistant *Staphylococcus aureus* strains Mu3 and
827 Mu50 by cDNA differential hybridization method. Biochem Biophys Res Commun
828 **269:**485-490.
829 42. **Boyle-Vavra S, Yin S, Jo DS, Montgomery CP, Daum RS.** 2013. VraT/YvqF is
830 required for methicillin resistance and activation of the VraSR regulon in
831 *Staphylococcus aureus*. Antimicrob Agents Chemother **57:**83-95.
832 43. **DeFrancesco AS, Masloboeva N, Syed AK, DeLoughery A, Bradshaw N, Li GW,**
833 **Gilmore MS, Walker S, Losick R.** 2017. Genome-wide screen for genes involved in
834 eDNA release during biofilm formation by *Staphylococcus aureus*. Proc Natl Acad Sci
835 U S A **114:**E5969-E5978.
836 44. **Vickery CR, Wood BM, Morris HG, Losick R, Walker S.** 2018. Reconstitution of
837 *Staphylococcus aureus* Lipoteichoic Acid Synthase Activity Identifies Congo Red as a
838 Selective Inhibitor. J Am Chem Soc **140:**876-879.
839 45. **Fey PD, Endres JL, Yajjala VK, Widhelm TJ, Boissy RJ, Bose JL, Bayles KW.**
840 2013. A genetic resource for rapid and comprehensive phenotype screening of
841 nonessential *Staphylococcus aureus* genes. MBio **4:**e00537-00512.
842 46. **Terrak M, Nguyen-Distèche M.** 2006. Kinetic characterization of the monofunctional
843 glycosyltransferase from *Staphylococcus aureus*. Journal of bacteriology **188:**2528-
844 2532.
845 47. **Rebets Y, Lupoli T, Qiao Y, Schirner K, Villet R, Hooper D, Kahne D, Walker S.**
846 2014. Moenomycin resistance mutations in *Staphylococcus aureus* reduce
847 peptidoglycan chain length and cause aberrant cell division. ACS Chem Biol **9:**459-
848 467.

849 48. **De Jonge B, Chang Y-S, Gage D, Tomasz A.** 1992. Peptidoglycan composition of a
850 highly methicillin-resistant Staphylococcus aureus strain. The role of penicillin binding
851 protein 2A. Journal of Biological Chemistry **267:**11248-11254.
852 49. **Reichmann NT, Picarra Cassona C, Monteiro JM, Bottomley AL, Corrigan RM,**
853 **Foster SJ, Pinho MG, Gründling A.** 2014. Differential localization of LTA synthesis
854 proteins and their interaction with the cell division machinery in *Staphylococcus*
855 *aureus*. Mol Microbiol **92:**273-286.
856 50. **Commichau FM, Gibhardt J, Halbedel S, Gundlach J, Stülke J.** 2017. A Delicate
857 Connection: c-di-AMP Affects Cell Integrity by Controlling Osmolyte Transport.
858 Trends in microbiology **26:**175-185.
859 51. **Corrigan RM, Campeotto I, Jeganathan T, Roelofs KG, Lee VT, Gründling A.**
860 2013. Systematic identification of conserved bacterial c-di-AMP receptor proteins.
861 Proceedings of the National Academy of Sciences **110:**9084-9089.
862 52. **Schuster CF, Bellows LE, Tosi T, Campeotto I, Corrigan RM, Freemont P,**
863 **Gründling A.** 2016. The second messenger c-di-AMP inhibits the osmolyte uptake
864 system OpuC in *Staphylococcus aureus*. Sci Signal **9:**ra81.
865 53. **Memmi G, Filipe SR, Pinho MG, Fu Z, Cheung A.** 2008. *Staphylococcus aureus*
866 PBP4 is essential for beta-lactam resistance in community-acquired methicillin-
867 resistant strains. Antimicrob Agents Chemother **52:**3955-3966.
868 54. **Gründling A, Schneewind O.** 2007. Genes required for glycolipid synthesis and
869 lipoteichoic acid anchoring in *Staphylococcus aureus*. J Bacteriol **189:**2521-2530.
870 55. **Covas G, Vaz F, Henriques G, Pinho M, Filipe S.** 2016. Analysis of Cell Wall
871 Teichoic Acids in *Staphylococcus aureus*. , p 201-213. *In* Hong H (ed), Bacterial Cell
872 Wall Homeostasis, vol 1440. Humana Press, New York, NY.

873 56. **Monk IR, Tree JJ, Howden BP, Stinear TP, Foster TJ.** 2015. Complete Bypass of
874 Restriction Systems for Major *Staphylococcus aureus* Lineages. MBio **6:**e00308-
875 00315.
876 57. **Zeden MS, Schuster CF, Bowman L, Zhong Q, Williams HD, Gründling A.** 2018.
877 Cyclic-di-adenosine monophosphate (c-di-AMP) is required for osmotic regulation in
878 *Staphylococcus aureus* but dispensable for viability in anaerobic conditions. J Biol
879 Chem doi:10.1074/jbc.M117.818716.
880 58. **Boles BR, Thoendel M, Roth AJ, Horswill AR.** 2010. Identification of genes involved
881 in polysaccharide-independent *Staphylococcus aureus* biofilm formation. PLoS One
882 **5:**e10146.
883