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and cellular morphology in a triclosan-induced small-
colony variant of staphylococcus aureus**

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1 **Altered Competitive Fitness, Antimicrobial Susceptibility**
2 **and Cellular Morphology in a Triclosan-Induced Small**
3 **Colony Variant of *Staphylococcus aureus***
4

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14

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31 **ABSTRACT**

32 *Staphylococcus aureus* can produce small colony variants (SCVs) which express various
33 phenotypes. Whilst their significance is unclear, SCV propagation may be influenced by
34 relative fitness, antimicrobial susceptibility and the underlying mechanism. We have
35 investigated triclosan-induced generation of SCVs in six *S. aureus* strains, including MRSA.
36 Parent strains (P0) were repeatedly passaged on concentration gradients of triclosan using a
37 solid-state exposure system to generate P10. P10 was subsequently passaged without
38 triclosan to generate X10. Susceptibility to triclosan and 7 antibiotics was assessed at all
39 stages. For *S. aureus* ATCC6538, SCVs were further characterised by determining
40 microbicide susceptibility and competitive fitness. Cellular morphology was examined using
41 electron microscopy and protein expression evaluated through proteomics. Triclosan
42 susceptibility in all SCVs (which could be generated from 4/6 strains) was markedly
43 decreased, whilst antibiotic susceptibility was significantly increased in the majority of cases.
44 A SCV of *S. aureus* ATCC6538 exhibited significantly increased susceptibility to all tested
45 microbicides. Cross-wall formation was impaired in this bacterium, whilst expression of
46 FabI, a target of triclosan and IsaA, a lytic transglycosylase involved in cell division, was
47 increased. The P10 SCV was 49% less fit than P0. In summary, triclosan exposure of *S.*
48 *aureus* produced SCVs in 4/6 test bacteria, with decreased triclosan susceptibility but with
49 generally increased antibiotic susceptibility. A SCV derived from *S. aureus* ATCC6538
50 showed reduced competitive fitness, potentially due to impaired cell division. In this SCV,
51 increased FabI expression could account for reduced triclosan susceptibility, whilst IsaA may
52 be upregulated in response to cell division defects.

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63 **INTRODUCTION**

64 *Staphylococcus aureus* small colony variants (SCVs) are characterised by low growth rate
65 and the formation of small non-pigmented colonies (1, 2). They are commonly, but not
66 exclusively, related to antibiotic exposure (3) and have been shown to display diverse
67 phenotypic characteristics, including reduced β -haemolysis, coagulase and DNase activity
68 (4), enhanced intracellular survival (5), impaired biofilm formation (6), reduced virulence (6)
69 and low intrinsic susceptibility to certain antibiotics, cationic microbicides and antimicrobial
70 peptides (7, 8). Whilst all SCVs are not physiologically the same, certain SCVs have been
71 reported to cause persistent skin, bone and device-associated infections and they have been
72 isolated from patients undergoing prolonged antibiotic therapy (2, 9, 10). Due to their
73 uncommon morphological features and pin-point colony size, SCVs may be overlooked or
74 misidentified in clinical microbiology laboratories, potentially confounding their
75 identification.

76 The phenotypic variation observed in *S. aureus* SCVs is often attributed to auxotrophy for
77 menadione, hemin or thiamine due to mutations in their respective genes. This results in
78 impaired synthesis of menaquinone and cytochromes causing defects in the electron transport
79 chain (1, 11, 12). A resulting reduction in transmembrane potential leads to a decrease in
80 ATP production, which may cause impaired cell wall synthesis as well as a decrease in
81 growth rate resulting in a smaller bacterial colony size (12, 13). This metabolic change can
82 also lead to alterations in pigmentation and exotoxin expression (5, 6, 14).

83

84 *S. aureus* SCVs recovered from clinical specimens taken from the bronchial secretions of
85 cystic fibrosis (CF) patients have displayed auxotrophy for thymidine due to mutations in
86 thymidylate synthase (*thyA*), an enzyme involved in thymidine synthesis through the
87 production of dTMP (9, 15). Recent studies reveal *thyA* mutants exhibit resistance to

88 trimethoprim–sulfamethoxazole, a common treatment for CF (15). Trimethoprim–
89 sulfamethoxazole inhibits tetrahydrofolic acid production, which is a cofactor for thymidylate
90 synthase thus involved in thymidine synthesis (16). It is therefore apparent that antibiotic
91 treatment may provide a selective pressure that favours the selection of SCVs.

92

93 A decrease in transmembrane potential in SCV electron transport-defective mutants may
94 result in reduced susceptibility to certain antibiotics and cationic microbicides due to a
95 reduction in cell wall metabolism, lower growth rate and impaired uptake of positively
96 charged molecules to the bacterial cell (17-19). For example, clinical SCV isolates of *S.*
97 *aureus* have previously shown reduced susceptibility to β -lactams and aminoglycosides (15,
98 17, 20). Furthermore, it has been suggested that SCVs may potentially gain a survival
99 advantage within the host by their ability to persist within phagocytes, due in part to a
100 decrease in α -toxin production, and are therefore shielded from host immune defences as well
101 as the actions of antibiotics (5, 21, 22). The clinical significance of these purported attributes
102 however, depends on whether the SCV can revert to full virulence following cessation of
103 treatment, which in turn depends upon the stability of the responsible mutations and the
104 relative fitness of the SCV when compared to the parent strain (23, 24).

105

106 Induction of the SCV phenotype in *S. aureus* after sub-effective exposure to triclosan has
107 been previously reported (6, 25, 26). Triclosan is a bisphenol microbicide that is often
108 incorporated into disinfectant washes, toothpastes, cosmetics and household products for the
109 purpose of antiseptis and disinfection (27-29). Triclosan exerts bacteriostatic activity through
110 inhibition of FabI, an enoyl-ACP reductase, which participates in fatty acid synthesis (30-32).
111 At higher concentrations triclosan is bactericidal due to direct effects on the cytoplasmic
112 membrane (33). Whilst resistance to in-use concentrations of microbicides is rare, certain

113 bacteria are reported to have developed a reduced susceptibility to triclosan after sub-
114 inhibitory exposure *in vitro* (6, 34, 35). This may be due to point mutations in the *fabI* gene
115 (36), overexpression of FabI or due to increased efflux pump activity leading to the removal
116 of the compound from the cell (37, 38). In *S. aureus*, sub-lethal exposure to triclosan has been
117 shown to induce the formation of triclosan insusceptible SCVs that display alterations in
118 metabolism, virulence and reduced susceptibility to gentamicin (6, 35, 39).

119

120 We have previously described the generation of a SCV in *S. aureus* ATCC 6538 in response
121 to repeated sub-lethal triclosan exposure (6) which displayed reduced susceptibility to
122 triclosan, lower growth rate, impaired biofilm formation and reduced pathogenicity when
123 compared to the parent strain. The current investigation evaluates the effect of triclosan on
124 the induction of the SCV state in 5 other strains of *S. aureus*, as well as further characterising
125 the phenotypic changes in the previously generated SCV with respect to susceptibility to
126 antibiotics and cationic microbicides, as well as alterations in competitive fitness, cellular
127 morphology and protein expression.

128

129 **MATERIALS AND METHODS**

130 **Chemical Reagents and Growth Media.** Bacteriological growth media were
131 purchased from Oxoid (Basingstoke, UK). Chemical reagents were purchased from Sigma-
132 Aldrich (Dorset, UK) unless otherwise stated. Vantocil (a 20% v/v aqueous solution of
133 PHMB) was obtained from Arch Chemicals Inc. (Manchester, UK).

134 **Bacterial strains and growth media.** *Staphylococcus aureus* strains ATCC 6538 and
135 ATCC 43300 (MRSA) were supplied by the American Type Culture Collection. Strains
136 Newman, NCTC 6571, NCTC 13277 (MRSA) and NCTC 13142 (MRSA) were obtained
137 from Public Health England (Salisbury, UK). Bacteria were grown on Tryptone Soya agar

138 (TSA) or Tryptone Soya broth (TSB). Cultures were incubated aerobically at 37°C for 18-24h
139 unless otherwise stated. Bacteria were archived at -80°C prior to triclosan exposure (parent
140 strain P0), after 10 passages across a triclosan gradient (P10; SCV) and after a further 10
141 passages in the absence of triclosan (X10).

142 **Selection of isolates with reduced triclosan susceptibility.** Reproducible
143 concentration gradients of triclosan were created on TSA agar by depositing stock solutions
144 of triclosan (100 µg/ml- 10 mg/ml) with a Wasp II spiral plater (Don Whitley, Shipley,
145 United Kingdom) (6). Plates were dried for 1 h at room temperature prior to radial deposition
146 of an overnight suspension of *S. aureus* and incubated for 4 days aerobically at 37°C. Growth
147 observed at the highest triclosan concentration was removed and used to inoculate further
148 gradient plates. This process was repeated for 10 passages. A further 10 passages were
149 performed on triclosan-free TSA. Isolates (the parent P0 strain), those passaged 10 times on
150 triclosan (strain P10), and those passaged a further 10 times on triclosan-free TSA (strain
151 X10) were archived at -80°C for subsequent analyses.

152 **Minimum inhibitory concentrations (MICs) and minimum bactericidal**
153 **concentrations (MBCs).** MIC values were determined using the micro dilution method as
154 described previously (40). Briefly, overnight bacterial cultures were adjusted to an OD₆₀₀ of
155 0.8 and diluted 1:100 in TSB to produce a bacterial inoculum for susceptibility testing.
156 Inocula were incubated with doubling dilutions of the relevant microbicide at 37°C for 24 h.
157 The MIC was defined as the lowest concentration for which bacterial growth did not occur.
158 Growth was defined as turbidity (496nm) in comparison to an uninoculated well (negative
159 control). Aliquots (10µl) from wells exhibiting no growth were transferred to sterile TSA and
160 incubated at 37°C. The MBC was defined as the lowest concentration of microbicide at which
161 no bacterial growth occurred after 4d of incubation.

162 **Disc diffusion tests.** Antibiotic susceptibilities were determined for ciprofloxacin
163 (1µg), cephalothin (30µg), ampicillin (10µg), kanamycin (10µg), tetracycline (10µg),
164 gentamicin (10µg) and Trimethoprim–sulfamethoxazole (25µg). Disc diffusion assays were
165 performed according to the standardized British Society for Antimicrobial Chemotherapy
166 (BSAC) disc diffusion method for antimicrobial susceptibility testing (41). Plates were
167 incubated for 48 h at 37°C.

168 **Protein extraction and isoelectric focusing.** Bacterial cultures were grown in TSB at
169 37°C and 100 rpm for 18h, diluted 1:100 and incubated at 37°C with shaking at 100rpm to
170 mid-log phase (OD₆₀₀ of 0.4). Cultures were pelleted at 12,000 x g, washed in PBS (0.01 M
171 phosphate buffer, 0.0027M potassium chloride and 0.137M sodium chloride, pH 7.4, 3 x
172 3ml) and resuspended in PBS (1ml). To extract protein, lysostaphin (50µg/ml) was added and
173 the suspensions were incubated for 15min on ice prior to sonication at an amplitude of 10µ in
174 6 x 30 second bursts. Protein was precipitated in a 1:1:8 solution of cell lysate with
175 trichloroacetic acid (6.1 N) and acetone and incubated at -20°C for 1h. Protein was pelleted
176 by centrifugation at 16,000 x g, washed three times in acetone (1ml) and dissolved in
177 rehydration buffer (9M urea, 2% CHAPS, 1% DTT, 2% carrier ampholytes, 0.5% protease
178 inhibitor, 0.001% bromophenol blue, 2 ml). Soluble protein concentration was quantified
179 using the Bradford assay (Sigma, Poole, UK). Between 250 µg and 500 µg of protein per 200
180 µl total volume of buffer was loaded per 11cm ReadyStrip™ immobilised protein gradient
181 (IPG) strip pH 5-8 (Bio-Rad, Hertfordshire, UK). Strips were rehydrated under active
182 conditions overnight using a PROTEAN IEF cell (Bio-Rad, Hertfordshire, UK). After
183 rehydration, isoelectric focusing was conducted as follows; 250V for 15 min, linear voltage
184 to 8000V, 500V until the run was completed.

185 **Two dimensional gel electrophoresis.** IPG strips were equilibrated using
186 equilibration buffer 1 (6M urea, 2% SDS, 50mM Tris-HCL pH 8.8, 2% glycerol and 1%

187 DTT, 5 ml) followed by equilibration buffer 2 (6M urea, 2% SDS, 50 mM Tris-HCL pH 8.8,
188 2% glycerol and 2.5% iodoacetamide, 5 ml). Polyacrylamide casting gels (34ml distilled
189 water, 25ml 1.5 M Tris-HCL pH 8.8, 0.5ml of 20% SDS and 40 ml of 30% bis-acrylamide)
190 were polymerised by the addition of 10% ammonium persulphate (0.5ml) and
191 tetramethylethylenediamene (TEMED) (100µl). Stacking gel solution (34 ml distilled water,
192 6.25ml of 1 M Tris-HCL, 0.25ml of 20% SDS, 8.5ml of 30% bis-acrylamide, 0.25 ml APS
193 and 50µl TEMED) was poured above the set casting gel and IPG strips were loaded above the
194 stacking gel. Gels were run at 20V for 1-2h, then at 55V for 15-18h before being fixed for 8h
195 (500ml ethanol, 400ml water and 100ml acetic acid) at room temperature and stained with a
196 coomassie blue stain (0.8g coomassie blue R350, 400 ml of 40% methanol and 400 ml 20%
197 acetic acid) for 18h at room temperature and 20rpm. After destaining, (500ml methanol,
198 400ml water and 100ml acetic acid) gel spots of interest were excised and proteins were
199 identified using tandem mass spectrometry, performed at The Biomolecular Analysis Facility
200 within The University of Manchester.

201 **Transmission electron microscopy.** Cultures (50ml) were grown to an OD₆₀₀ of 0.3
202 in TSB and bacterial cells were pelleted via centrifugation at 16,000 x g for 10min. Cells
203 were resuspended in 0.25% gluteraldehyde (1ml) at 4°C, further fixed in 2% osmium
204 tetroxide and passed through an ethanol dehydration series. Cells were sectioned (80nm) and
205 TEM was conducted using a FEI Polara 300kV FEG transmission electron microscope (FEI,
206 Oregon, USA) at The University of Manchester imaging suite.

207 **Competitive fitness assay.** Competitive fitness was assessed using methods outlined
208 previously (42). Overnight cultures of *S. aureus* P0 or P10 were diluted 1:10 and adjusted to
209 an OD₆₀₀ of 1.5. Sterile TSB (250ml) was inoculated in triplicate with P0 or P10, alone or in
210 combination (final inoculum volume, 500µl). Flasks were incubated at 37°C with shaking at
211 100 rpm for 24 h. At 0 h and 24 h, dilutions from each flask (10^{-2} to 10^{-6}) were plated onto

212 TSA and TSA containing $1\mu\text{g ml}^{-1}$ triclosan (TSA_{TCS}) in triplicate and incubated at 37°C for
213 18h. Bacterial viable counts were determined after 18h of incubation, and relative fitness was
214 assessed for bacteria grown independently and in combination, using the equation; $W = \ln$
215 $(\text{RF}/\text{RI}) / \ln (\text{SF}/\text{SI})$ where W refers to relative fitness, RI and SI refer to the number of SCV
216 and susceptible cells at the start point, respectively and RF and SF to the number of SCV and
217 susceptible cells at endpoint.

218

219 **RESULTS**

220 **Altered triclosan and antibiotic susceptibility in triclosan-exposed *S. aureus*.** In
221 addition to the SCV (R1), previously induced by the exposure of *S. aureus* ATCC 6538 to
222 triclosan (6), SCVs were similarly formed by the replicate triclosan-exposure of *S. aureus*
223 ATCC 6538 (R2), as well as by strains Newman, ATCC 43300 and NCTC 13277. Colony
224 morphology in *S. aureus* strains NCTC 6571 and NCTC 13142 however, remained
225 unchanged after repeated triclosan exposure.

226 Triclosan susceptibility (MIC and MBC) significantly decreased in all P10 strains (SCV and
227 non-SCV) when compared to the respective parent strains (Table 1 $P < 0.01$). After passage in
228 the absence of triclosan (X10) MICs and MBCs frequently partially reverted but remained
229 significantly higher than the pre-exposure values for all test strains ($P < 0.01$). When
230 comparing the susceptibility of the P0 to P10 strains, for SCVs, increases in MIC ranged
231 from 4 to 31-fold whilst increases in MBC ranged from 3 to 16-fold. In comparison, for non-
232 SCV strains MICs increased from 5 to 11-fold whilst MBCs increased from 4 to 8-fold.

233 In terms of antibiotic susceptibility, our previously formed *S. aureus* ATCC 6538 R1 showed
234 a significant increase in sensitivity to all test antibiotics with the exception of ampicillin,
235 when compared to the parent strain (Table 2; $P < 0.05$). Antibiotic susceptibilities partially
236 reverted to pre-exposure values when the SCV was allowed to recover in the absence of

237 triclosan. However, susceptibilities of X10 remained significantly higher than the parent
238 strain for cephalothin, gentamicin, kanamycin, and thiomethoprim-sulfamethoxazole
239 ($P<0.05$). Replicate SCV strain ATCC 6538 R2 also exhibited a significant increase in
240 susceptibility to cephalothin, gentamicin and tetracycline, which remained elevated in the
241 absence of triclosan for cephalothin and gentamicin (X10; $P<0.05$). *S. aureus* ATCC 43300
242 (SCV) increased in susceptibility to all test antibiotics with the exception of gentamicin,
243 kanamycin and ciprofloxacin however, all increases in susceptibility fully or partially
244 reverted back to pre-exposure levels once the bacteria were passaged without triclosan (X10;
245 $P<0.05$). *S. aureus* NCTC 13277 (SCV) was more susceptible to gentamicin and
246 trimethoprim-sulfamethoxazole after triclosan exposure, whilst X10 strains showed no
247 significant difference in susceptibility when compared to the unexposed parent strain (P0). *S.*
248 *aureus* Newman (SCV) exhibited increased susceptibility to ciprofloxacin, cephalothin,
249 kanamycin, gentamicin and a decrease in trimethoprim-sulfamethoxazole susceptibility;
250 however X10 strains only showed a significantly different susceptibility than P0 to
251 trimethoprim-sulfamethoxazole and cephalothin. In non-SCV forming strains, NCTC 6571
252 exhibited a significant increase in cephalothin susceptibility after repeated triclosan exposure,
253 whilst NCTC 13142 showed a reduction in trimethoprim-sulfamethoxazole susceptibility,
254 neither of which fully reverted to pre-exposure levels in the absence of triclosan, ($P<0.05$).

255 **Two-dimensional (2D) gel electrophoresis of a parent and triclosan-exposed**
256 **strain of *S. aureus* ATCC 6538 revealed differences in protein expression.** Proteins of
257 interest were identified using tandem mass spectrometry (MS-MS) after electrospray
258 ionisation (Figure 1A-B). Notably, upregulation of triclosan target enzyme FabI was
259 observed in the SCV strain. There was an evident increase in peptide deformylase (Def)
260 production after triclosan exposure, which is a participant in protein synthesis in bacteria. A
261 possible increase in expression of transglycosylase IsaA, an autolysin involved in cell wall

262 cleavage during cell replication, was also detected in the SCV strain.

263 **A triclosan-adapted *S. aureus* ATCC 6538 SCV exhibits abnormal cell**
264 **morphology.** The internal cellular morphologies of *S. aureus* parent strain (P0), SCV (P10)
265 and recovered X10 strain were visualised using TEM (Figure 2). High-resolution
266 micrographs revealed that the SCV exhibited a higher frequency of irregular-shaped or
267 abnormally dividing cells due to asymmetrical septum formation. The mean diameter of the
268 SCV cells were on average 32.8% and 28.3% greater than those of the P0 or X10 strain
269 respectively ($P < 0.001$). There was no significant difference between the diameters of P0 and
270 X10.

271 **Altered cationic microbicide susceptibility in a triclosan induced *S. aureus***
272 **ATCC 6538 SCV.** When compared to P0, MICs for polyhexamethylene biguanide (PHMB),
273 chlorhexidine and benzalkonium chloride significantly decreased in the *S. aureus* SCV (P10)
274 from 3.6 $\mu\text{g/ml}$ to 1.8 $\mu\text{g/ml}$ for benzalkonium chloride and chlorhexidine and from 15.6
275 $\mu\text{g/ml}$ to 3.6 $\mu\text{g/ml}$ for PHMB ($P < 0.001$; Table 3). MBCs of both the biguanides were also
276 significantly decreased in P10 from 93.8 $\mu\text{g/ml}$ to 31.6 $\mu\text{g/ml}$ for PHMB and from 15.6
277 $\mu\text{g/ml}$ to 7.8 $\mu\text{g/ml}$ for chlorhexidine. The MBC for Benzalkonium chloride (BAC) did not
278 change between P0 and P10 (Table 3). After passage in the absence of triclosan (X10), the
279 MIC of PHMB partially reverted to the pre-exposure level, whereas MICs of chlorhexidine
280 and BAC did not revert. In terms of bactericidal activity, MBCs of PHMB and chlorhexidine
281 fully reverted to pre-exposure levels in the absence of any microbicide (X10).

282 **Reduced competitive fitness of a *S. aureus* ATCC 6538 SCV compared to the**
283 **parent strain.** The overall productivity (cfu/ml) of P10 after 24 h of growth was significantly
284 lower than that of P0. This deficit in growth was substantially more pronounced when the
285 strains were grown in competition (Figure 3). The relative Darwinian fitness (W) of P0 and
286 P10 was compared when grown separately and when in competition with each other (Figure

287 3). By definition a relative fitness of 1 indicates no fitness effect between strains, a value of
288 below 1 implies impaired fitness and above 1 enhanced fitness (42). The relative fitness (W)
289 of P10 to P0 during individual growth was 0.97, compared to 0.51 during competition.
290 Therefore, in a non-competitive environment P10 grew 3% slower than P0, whereas when in
291 a competitive environment P10 grew 49% slower than P0.

292

293 **DISCUSSION**

294 In the current investigation, the repeated exposure of *S. aureus* to triclosan selected for
295 substantially reduced triclosan susceptibility in 6/6 test strains whilst only 4/6 formed the
296 SCV phenotype. In SCVs, antibiotic susceptibility significantly increased in 3/5 strains for
297 tetracycline, 2/5 for kanamycin, 5/5 for gentamicin, 3/5 for trimethoprim-sulfamethoxazole,
298 1/5 for ampicillin, 2/5 for ciprofloxacin and 4/5 for cephalothin. The only decrease in
299 antibiotic susceptibility observed in a SCV was in *S. aureus* Newman for trimethoprim-
300 sulfamethoxazole, which reverted in the absence of triclosan. In the two non-SCV forming
301 strains, only NCTC 6571 showed a significant increase in antibiotic susceptibility after
302 triclosan exposure (to cephalothin), whilst non-SCV forming strain NCTC 13142 showed a
303 significant decrease in trimethoprim-sulfamethoxazole susceptibility. *S. aureus* strain ATCC
304 6538 SCV R1 exhibited the largest increase in both triclosan and antibiotic susceptibility
305 when compared to the P0 and was therefore further evaluated for alterations in protein
306 expression, competitive fitness, cationic microbicide susceptibility and cellular morphology.

307 Proteomic analysis of the *S. aureus* ATCC 6538 SCV R1 (P10) and parent strain (P0)
308 revealed changes in protein expression after repeated sub-lethal triclosan exposure, notably
309 an upregulation of triclosan target enzyme FabI, which may explain previously observed
310 decreases in triclosan susceptibility (6). An increase in the expression of peptide deformylase,

311 Def, a metalloenzyme involved in protein synthesis, may indicate an overall elevation in
312 protein synthesis in the SCV (43), possibly as part of a generalised stress response. An
313 increase in IsaA expression was also observed in this SCV strain. A major role of this
314 enzyme is the hydrolysis of bonds within peptidoglycan thus allowing cell wall expansion
315 and cell growth (44). TEM analysis of cell morphology revealed a high proportion of SCV
316 cells with an abnormal shape and impaired septation, resulting in significantly larger cells
317 than the parent (P0) and the partly recovered (X10) strains. It is therefore possible that the
318 over-expression of IsaA may occur in response to this morphological defect, in an attempt to
319 compensate for the lack of cell division observed in this SCV strain. Both thymidine and
320 haemin auxotrophic SCVs have previously presented as enlarged cocci with multiple cross
321 walls when viewed using scanning electron microscopy (13), which is consistent with the
322 impaired cell septation observed in the current SCV. However, previous analysis of this SCV
323 strain revealed no auxotrophy for thymidine or haemin (6). This defective cell division may
324 help further account for the reduced growth rate and small colony size of the SCV when
325 compared to the parent (P0) strain.

326 The generation of *S. aureus* SCVs by exposure to various antimicrobials, has been previously
327 associated with decreased susceptibility to certain antibiotics, cationic microbicides and
328 recently, to human antimicrobial peptides (8, 45). This reduction in susceptibility is often
329 attributed to defects in the electron transport chain, as well as reduced growth rates and
330 hypermutability (11, 16, 19). In contrast, in the present study a triclosan-induced SCV in *S.*
331 *aureus* strain ATCC 6538 (R1) exhibited increased susceptibility to 6/7 test antibiotics and to
332 the cationic microbicides PHMB, chlorhexidine and BAC. Interestingly, the only antibiotic to
333 which this SCVs susceptibility did not increase significantly towards was ampicillin, a
334 transpeptidase inhibitor that interferes with bacterial cell wall formation. TEM revealed
335 impaired cross-wall formation in the SCV strain and proteomic analysis suggested an

336 increase in expression of IsaA a lytic transglycoylase involved in the hydrolysis of
337 peptidoglycan and thus cell wall expansion, turnover and cell growth (44). The
338 overexpression of IsaA may represent an adaptation to this functional deficit. Such
339 phenotypic compensation may reduce the effectiveness of ampicillin, potentially ameliorating
340 susceptibility increases in this SCV. Impairments in cell wall synthesis leading to a possible
341 increase in cell wall permeability may further explain why P10 was more susceptible to the
342 majority of antibiotics, as well as to the membranotropic cationic microbicides.

343 When comparing the relative fitness of the parent (P0) and SCV (P10) strains in *S. aureus*
344 ATCC 6538 SCV (R1), P10 grew at a 3% lower rate than P0 when grown independently, but
345 was 49 % slower when grown in competition, highlighting the competitive advantage of the
346 P0 strain. The impaired ability of this SCV to undergo cell division may in part help account
347 for this reduced relative fitness. Previous investigations have demonstrated fitness costs
348 associated with antimicrobial resistance (23, 46, 47). It has been theorised that the fitness of a
349 bacterium is directly proportional to its rate of transmission and ability to compete with other
350 strains within the host, and this may be inversely proportional to its rate of clearance from the
351 host (48). Therefore, fitness however measured, may be an important predictor of the clinical
352 significance and potential for environmental persistence of a bacterium. For example, a
353 bacterium acquiring a mutation that results in antimicrobial resistance but also in a fitness
354 burden, may not establish in its environment due to reduced competitive fitness.
355 Alternatively, the bacterium could persist at low level for a prolonged period. However, if an
356 adapted bacterium cannot compete with its congeners or has a markedly reduced specific
357 growth rate then its pathogenic capability may be reduced (23). When grown in binary
358 culture with the mother strain, the triclosan-induced SCV in *S. aureus* ATCC 6538 (R1) in
359 the current investigation was outcompeted indicating the functional implications of
360 adaptation.

361 **CONCLUSION**

362 Repeated exposure to triclosan may select for SCVs in *S. aureus* exhibiting reduced triclosan
363 susceptibility but significantly increased susceptibility to certain antibiotics. In an SCV
364 generated from *S. aureus* ATCC 6538, reduced triclosan susceptibility may be partly
365 attributed to the overexpression of target enzyme FabI. This SCV also exhibited impaired
366 competitive fitness which may be due to defective cell division and an associated reduction in
367 planktonic growth. Additionally, this SCV strain showed increased susceptibility towards 6/7
368 test antibiotics and all tested cationic microbicides. Unlike previous reports, the formation of
369 the SCV phenotype in triclosan exposed *S. aureus* ATCC 6538, appears not to be due to
370 defects in haemin, menadione or thymidine synthesis but possibly due to impairment in cell
371 wall formation.

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536 TABLE 1. Triclosan susceptibility of *Staphylococcus aureus* before, during and after
 537 repeated triclosan exposure

| Test bacterium | MIC | | | MBC | | |
|--|-------|---------------|---------------|--------|----------------|----------------|
| | P0 | P10 | X10 | P0 | P10 | X10 |
| <i>Staphylococcus aureus</i> ATCC 6538 R1* | 1 | 31 | 7 | 4 | 63 | 14 (4) |
| <i>Staphylococcus aureus</i> ATCC 6538 R2* | 2 (1) | 21 (8) | 14 (4) | 8 (3) | 94 (34) | 29 (6) |
| <i>Staphylococcus aureus</i> NCTC 6571 | 2 | 21 (8) | 16 | 17 (6) | 63 | 31 |
| <i>Staphylococcus aureus</i> Newman* | 4 | 16 | 16 | 31 | 125 | 63 |
| <i>Staphylococcus aureus</i> ATCC 43300* | 2 | 16 | 14 (3) | 21 (6) | 63 | 41 (17) |
| <i>Staphylococcus aureus</i> NCTC 13277* | 4 | 18 (6) | 14 (3) | 16 | 63 | 57 (13) |
| <i>Staphylococcus aureus</i> NCTC 13142 | 4 | 18 (6) | 16 | 16 | 125 | 47 (17) |

538 Mean minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC)
 539 ($\mu\text{g/ml}$) of *S. aureus* before triclosan exposure (P0), after sub-lethal triclosan exposure (P10) and after
 540 recovery in a triclosan-free environment (X10). Data show duplicate experiments with three technical
 541 replicates. When data varied between replicates standard deviations are given in the parenthesis. Bold
 542 text indicates a statistically significant difference ($P < 0.001$) in MIC or MBC compared to that of the
 543 parent strain (P0). *Indicates strains that formed SCV after triclosan exposure. R1 and R2 indicate
 544 replicates 1 and 2 respectively.

545 TABLE 2. Antibiotic susceptibility in *Staphylococcus aureus* before, during and after repeated triclosan exposure

| <i>S. aureus</i> | TET | | | KAN | | | GEN | | | SXT | | | AMP | | | CIP | | | CEF | | |
|------------------|--------|---------------|---------------|--------|---------------|---------------|---------------|---------------|---------------|--------|---------------|---------------|--------|---------------|---------------|--------|---------------|--------|--------|---------------|---------------|
| | P0 | P10 | X10 | P0 | P10 | X10 | P0 | P10 | X10 | P0 | P10 | X10 | P0 | P10 | X10 | P0 | P10 | X10 | P0 | P10 | X10 |
| ATCC 6538 R1* | 26 (1) | 29 (1) | 25 (1) | 20 (1) | 29 (1) | 25 (1) | 21 (1) | 29 (1) | 24 (1) | 29 (1) | 37 (1) | 33 (1) | 44 (1) | 46 (3) | 46 (2) | 24 (2) | 27 (2) | 23 (1) | 43 (1) | 45 (1) | 44 (1) |
| ATCC 6538 R2* | 27 (1) | 30 (1) | 28 (2) | 18 (1) | 19 (1) | 19 (1) | 21 (1) | 26 (1) | 24 (1) | 28 (1) | 27 (1) | 26 (2) | 45 (3) | 48 (4) | 48 (1) | 25 (2) | 24 (2) | 23 (3) | 43 (1) | 47 (1) | 45 |
| NCTC 6571 | 25 (3) | 25 (4) | 26 | 18 (2) | 17 (2) | 17 (1) | 22 (3) | 22 (1) | 21 (1) | 28 (3) | 26 (2) | 27 (1) | 44 (4) | 42 (2) | 43 (1) | 28 (3) | 28 (2) | 27 (1) | 37 (1) | 43 (1) | 41 (1) |
| NEWMAN* | 28 (2) | 29 (1) | 26 (1) | 14 (3) | 20 (1) | 16 | 25 (3) | 29 (2) | 23 (1) | 20 (1) | 18 (1) | 22 (1) | 18 (2) | 19 (1) | 18 (1) | 26 (2) | 30 (1) | 23 (2) | 33 (1) | 37 (1) | 35 (1) |
| ATCC 43300* | 25 (1) | 32 (1) | 30 (1) | 0 | 0 | 0 | 9 (1) | 32 (1) | 28 (1) | 26 (3) | 30 | 26 (1) | 15 (2) | 22 (1) | 18 (1) | 22 (1) | 21 (1) | 22 (1) | 27 (1) | 48 (1) | 46 (1) |
| NCTC 13277* | 31 (4) | 30 (1) | 28 (1) | 0 | 0 | 0 | 22 (4) | 29 (1) | 23 (2) | 27 (4) | 29 (1) | 25 (1) | 11 | 11 (1) | 10 (1) | 0 | 0 | 0 | 0 | 0 | 0 |
| NCTC 13142 | 27 (3) | 28 (1) | 28 (1) | 15 (4) | 15 (2) | 16 (1) | 23 (1) | 22 (2) | 23 (2) | 29 (2) | 25 (2) | 25 (1) | 12 (2) | 13 (1) | 13 | 20 (4) | 23 (1) | 23 (2) | 28 (1) | 28 (1) | 28 (1) |

546 Antibiotic disc diffusion zones of inhibition (mm) of *S. aureus* before triclosan exposure (P0), after sub-lethal triclosan exposure (P10) and after recovery in a triclosan-free environment (X).
 547 TET, tetracycline; KAN, kanamycin; GEN, gentamicin; SXT, trimethoprim-sulfamethoxazole; AMP, ampicillin; CIP, ciprofloxacin; CEF, cephalothin Data show duplicate experiments each
 548 with three technical replicates. When data varied between replicates standard deviations are given in the parenthesis. Bold text indicates a statistical difference (P<0.05) in inhibition zone size
 549 when compared to that of the parent strain (P0). *Indicates strains that formed SCV after triclosan exposure (P10).

550 TABLE 3. The susceptibility of *Staphylococcus aureus* ATCC 6538 to cationic microbicides
 551 before, during and after repeated triclosan exposure

| Microbicide | MIC | | | MBC | | |
|-----------------------------|------|------------|------------|-----------|-------------|-----------|
| | P0 | P10 | X10 | P0 | P10 | X10 |
| Benzalkonium chloride | 3.6 | 1.8 | 1.8 | 15.6 | 15.6 | 15.6 |
| Chlorhexidine | 3.6 | 1.8 | 1.8 | 15.6 | 7.8 | 15.6 |
| Polyhexamethylene biguanide | 15.6 | 3.6 | 7.8 | 93.8 (34) | 31.2 | 93.8 (34) |

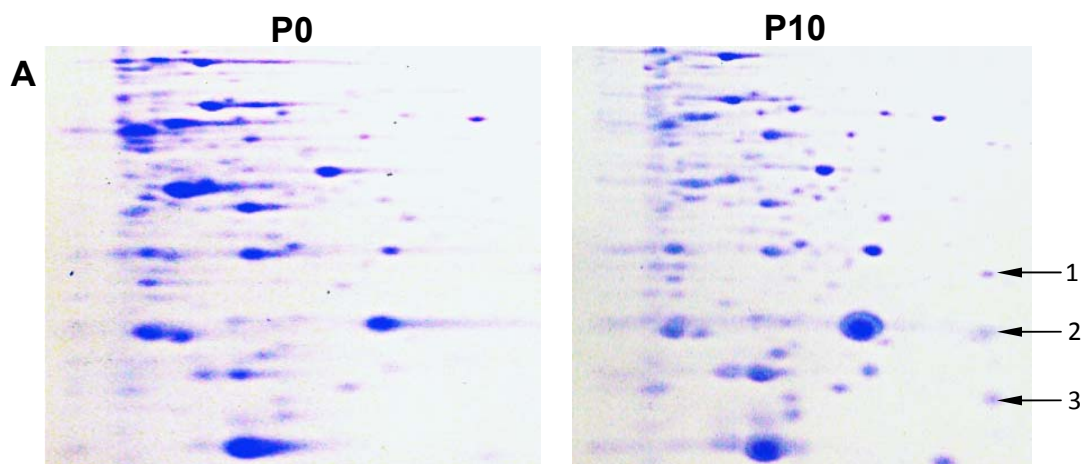
552 Mean minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC)
 553 ($\mu\text{g/ml}$) of *S. aureus* ATCC 6538 before triclosan exposure (P0), after sub-lethal triclosan exposure
 554 (P10; SCV) and after recovery in a triclosan-free environment (X10). Data show duplicate
 555 experiments with three technical replicates. When data varied between replicates standard deviations
 556 are given in the parenthesis. Bold text indicates a statistically significant difference ($P < 0.001$) in MIC
 557 or MBC compared to that of the parent strain (P0).

558

559 **FIG. 1**

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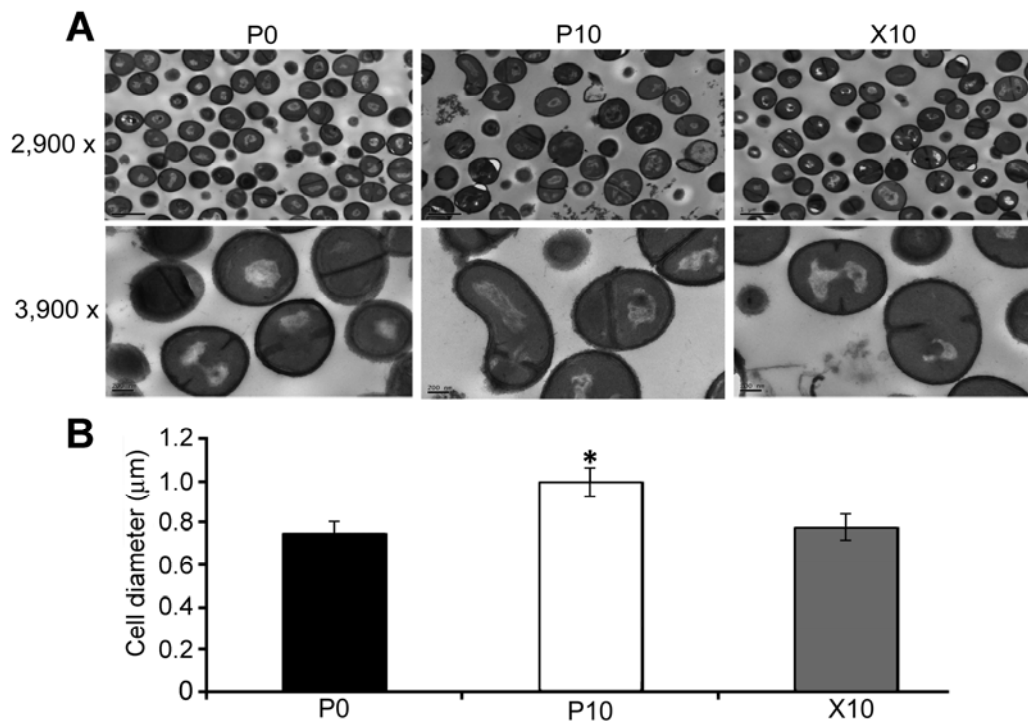
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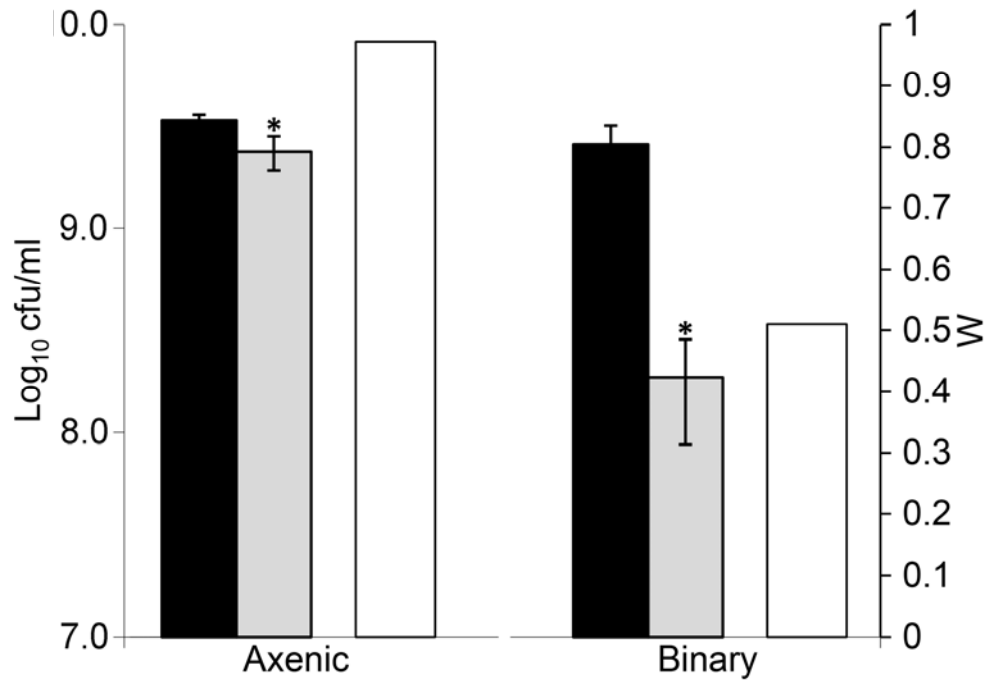
| Spot | Identity | Function | Accession no. |
|------|----------|---|---------------|
| 1 | FabI | Enoyl-[acyl-carrier-protein] reductase involved in fatty acid synthesis | FABI_STAAR |
| 2 | IsaA | Transglycosylase involved in the cleavage of peptidoglycan. | ISAA_STAA1 |
| 3 | Def | Peptide deformylase involved in protein translation. | DEF_STAAC |

FIG. 1. A) 2D gels showing protein expression profiles in P0 and P10 strains of *S. aureus* ATCC 6538. Proteins of interest were excised and identified using esi MS-MS. Indicated proteins were identified as FabI (1) IsaA (2) and Def (3). B) Identities and functions of proteins selected from 2D gels of *S. aureus* ATCC 6538 that were upregulated in triclosan induced SCVs.

FIG. 2

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564 **FIG. 2.** A) Cellular morphology of *S. aureus* ATCC 6538 parent strain P0, P10 and X10
565 strains visualised by TEM. B) Mean cell diameter P0 (black), P10 (white) and X10 (grey).
566 The asterisk indicates the significant difference in cell diameter of P10 when compared to P0
567 or X10 ($P < 0.001$).

FIG. 3



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569 **FIG 3.** Competitive fitness of P0 vs P10 (SCV) in *S. aureus* ATCC 6538. Black and grey
570 bars show cfu/ml of P0 and P10, respectively after 24h of growth, axenically or in binary
571 culture. White bars indicate relative fitness (W) under axenic and binary growth. Data are
572 means and standard deviations from four separate experiments with three technical replicates.
573 Error bars show standard deviation. Asterisks indicate statistically significant differences
574 ($P < 0.001$) compared to the parent strain (P0).

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