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

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Running title: Characterisation of a triclosan-induced small colony variant of *Staphylococcus aureus.*

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ABSTRACT

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

INTRODUCTION

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

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

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

3

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 antisepsis 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 OD_{600} 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 37ºC 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 37ºC. 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 (OD_{600} 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[TM] 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%

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

Transmission electron microscopy. Cultures (50ml) were grown to an OD_{600} of 0.3 in TSB and bacterial cells were pelleted via centrifugation at 16,000 x g for 10min. Cells were resuspended in 0.25% gluteraldehyde (1ml) at 4˚C, further fixed in 2% osmium tetroxide and passed through an ethanol dehydration series. Cells were sectioned (80nm) and TEM was conducted using a FEI Polara 300kV FEG transmission electron microscope (FEI, Oregon, USA) at The University of Manchester imaging suite.

Competitive fitness assay. Competitive fitness was assessed using methods outlined previously (42). Overnight cultures of *S. aureus* P0 or P10 were diluted 1:10 and adjusted to an OD_{600} of 1.5. Sterile TSB (250ml) was inoculated in triplicate with P0 or P10, alone or in combination (final inoculum volume, 500µl). Flasks were incubated at 37˚C with shaking at 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 1µg 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 (RF/RI)/ ln (SF/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

9

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 µg/ml to 1.8 µg/ml for benzalkonium chloride and chlorhexidine and from 15.6

275 µg/ml to 3.6 µg/ml for PHMB (P<0.001; Table 3). MBCs of both the biguanides were also

276 significantly decreased in P10 from 93.8 µg/ml to 31.6 µg/ml for PHMB and from 15.6

277 µg/ml to 7.8 µg/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,

12

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 membranotopic 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.

372
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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
Staphylococcus aureus ATCC 6538 R1*	1	**31**	**7**	4	**63**	**14 (4)**
Staphylococcus aureus ATCC 6538 R2*	2 (1)	**21 (8)**	**14 (4)**	8 (3)	**94 (34)**	**29 (6)**
Staphylococcus aureus NCTC 6571	2	**21 (8)**	**16**	17 (6)	**63**	**31**
Staphylococcus aureus Newman*	4	**16**	**16**	31	**125**	**63**
Staphylococcus aureus ATCC 43300*	2	**16**	**14 (3)**	21 (6)	**63**	**41 (17)**
Staphylococcus aureus NCTC 13277*	4	**18 (6)**	**14 (3)**	16	**63**	**57 (13)**
Staphylococcus aureus NCTC 13142	4	**18 (6)**	**16**	16	**125**	**47 (17)**

538 Mean minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC)
539 (µg/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

S. aureus	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   (µg/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

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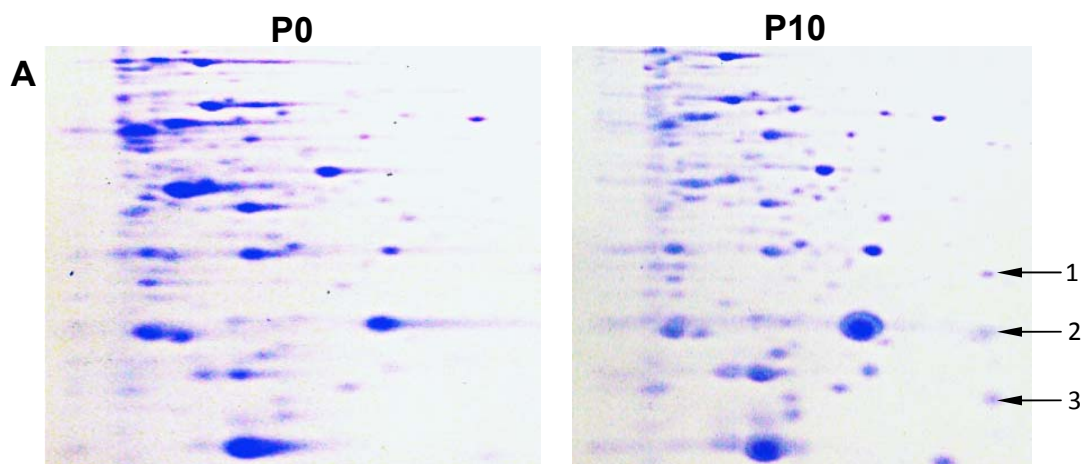
| Spot	Identity	Function	Accession no.
1	FabI	Enoyl-[acyl-carrier-protein] reductase involved in fatty acid synthesis	FABI_STAAR
2	IsaA	Transglycolsylase involved in the cleavage of peptidoglycan.	ISAA_STAA1
3	Def	Peptide deformylase involved in protein translation.	DEF_STAAC

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.
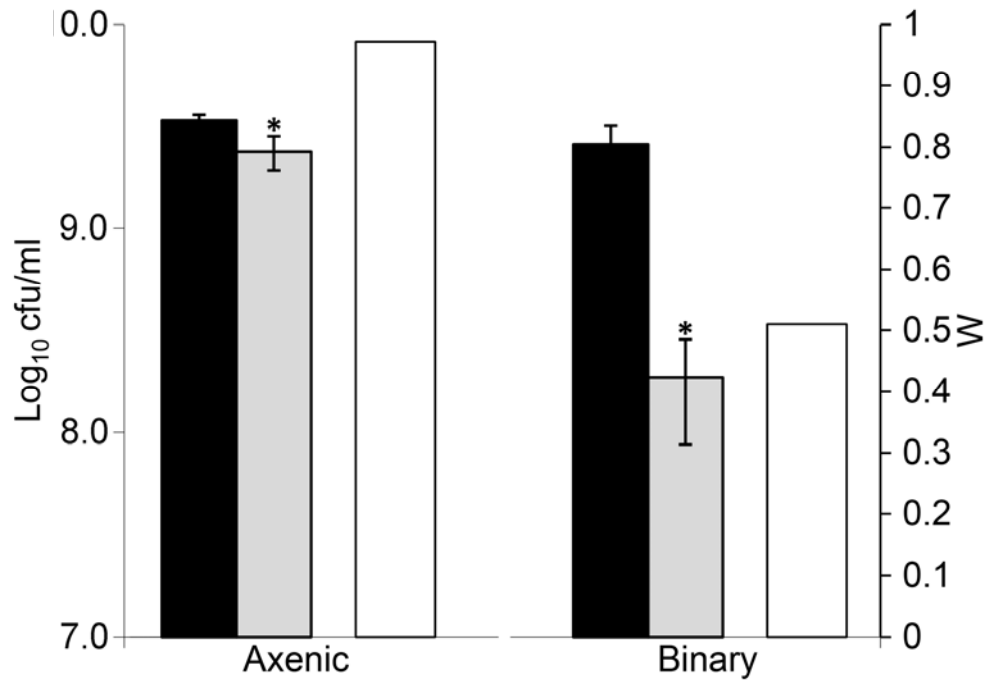
562
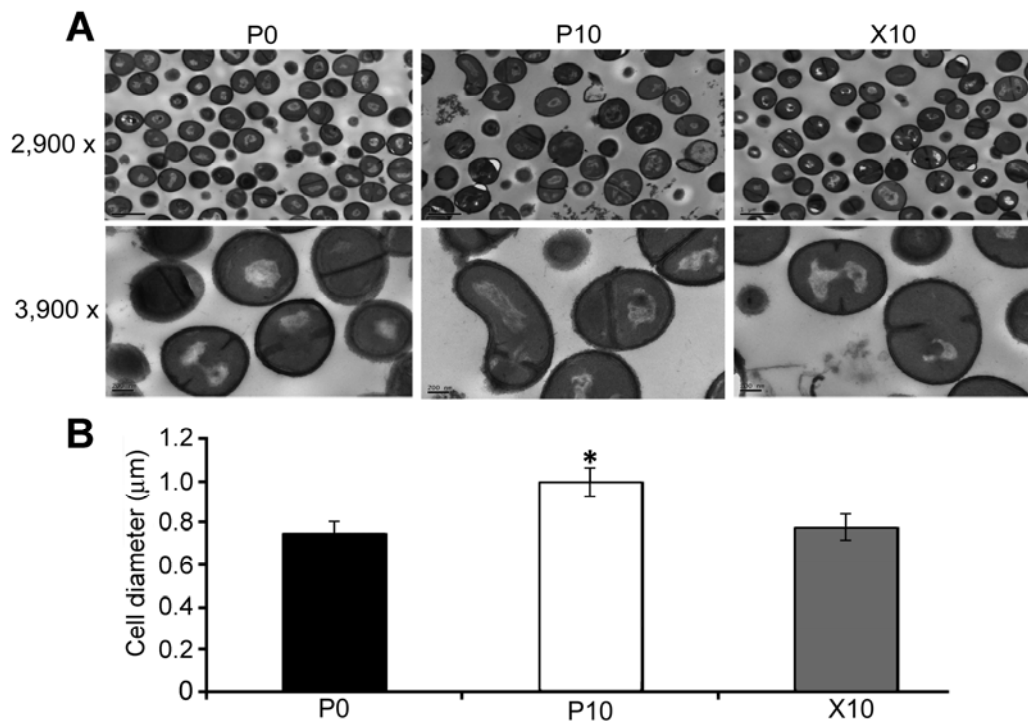
FIG. 2

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

FIG. 3

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