Sheffield Hallam University

Altered competitive fitness, antimicrobial susceptibility, and cellular morphology in a triclosan-induced small-colony variant of staphylococcus aureus

FORBES, Sarah <http://orcid.org/0000-0002-8361-6390>, LATIMER, Joe, BAZAID, Abdulrahman and MCBAIN, Andrew J.

Available from Sheffield Hallam University Research Archive (SHURA) at:

http://shura.shu.ac.uk/14499/

This document is the author deposited version. You are advised to consult the publisher's version if you wish to cite from it.

Published version

FORBES, Sarah, LATIMER, Joe, BAZAID, Abdulrahman and MCBAIN, Andrew J. (2015). Altered competitive fitness, antimicrobial susceptibility, and cellular morphology in a triclosan-induced small-colony variant of staphylococcus aureus. Antimicrobial Agents and Chemotherapy, 59 (8), 4809-4816.

Copyright and re-use policy

See http://shura.shu.ac.uk/information.html

Sheffield Hallam University Research Archive
http://shura.shu.ac.uk

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

Sarah Forbes*, Joe Latimer* Abdulrahman Bazaid and Andrew J. McBain[1#]

Manchester Pharmacy School, Stopford Building,
The University of Manchester, Manchester, UK.

Key Words: *Staphylococcus aureus*, triclosan, antibiotic, small colony variant, competitive fitness, septation, biocide, microbicide, proteomics.

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

[#]***For correspondence:*** Andrew McBain, Manchester Pharmacy School, The University of Manchester, Oxford Road, Manchester M13 9PT, UK. Tel: 00 44 161 275 2360; Fax: 00 44 161 275 2396; Email: andrew.mcbain@manchester.ac.uk

*****These authors contributed equally to this work

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs). MIC values were determined using the micro dilution method as described previously (40). Briefly, overnight bacterial cultures were adjusted to an OD_{600} of 0.8 and diluted 1:100 in TSB to produce a bacterial inoculum for susceptibility testing. Inocula were incubated with doubling dilutions of the relevant microbicide at 37ºC for 24 h. The MIC was defined as the lowest concentration for which bacterial growth did not occur. Growth was defined as turbidity (496nm) in comparison to an uninoculated well (negative control). Aliquots (10µl) from wells exhibiting no growth were transferred to sterile TSA and incubated at 37ºC. The MBC was defined as the lowest concentration of microbicide at which no bacterial growth occurred after 4d of incubation.

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

Protein extraction and isoelectric focusing. Bacterial cultures were grown in TSB at 37˚C and 100 rpm for 18h, diluted 1:100 and incubated at 37˚C with shaking at 100rpm to mid-log phase (OD_{600} of 0.4). Cultures were pelleted at 12,000 x g, washed in PBS (0.01 M phosphate buffer, 0.0027M potassium chloride and 0.137M sodium chloride, pH 7.4, 3 x 3ml) and resuspended in PBS (1ml). To extract protein, lysostaphin (50µg/ml) was added and the suspensions were incubated for 15min on ice prior to sonication at an amplitude of 10µ in 6 x 30 second bursts. Protein was precipitated in a 1:1:8 solution of cell lysate with trichloroacetic acid (6.1 N) and acetone and incubated at -20˚C for 1h. Protein was pelleted by centrifugation at 16,000 x g, washed three times in acetone (1ml) and dissolved in rehydration buffer (9M urea, 2% CHAPS, 1% DTT, 2% carrier ampholytes, 0.5% protease inhibitor, 0.001% bromophenol blue, 2 ml). Soluble protein concentration was quantified using the Bradford assay (Sigma, Poole, UK). Between 250 µg and 500 µg of protein per 200 µl total volume of buffer was loaded per 11cm ReadyStrip^{TM} immobilised protein gradient (IPG) strip pH 5-8 (Bio-Rad, Hertfordshire, UK). Strips were rehydrated under active conditions overnight using a PROTEAN IEF cell (Bio-Rad, Hertfordshire, UK). After rehydration, isoelectric focusing was conducted as follows; 250V for 15 min, linear voltage to 8000V, 500V until the run was completed.

Two dimensional gel electrophoresis. IPG strips were equilibrated using 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

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

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

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

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

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

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

DISCUSSION

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

Proteomic analysis of the *S. aureus* ATCC 6538 SCV R1 (P10) and parent strain (P0) revealed changes in protein expression after repeated sub-lethal triclosan exposure, notably an upregulation of triclosan target enzyme FabI, which may explain previously observed 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

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

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

CONCLUSION

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

REFERENCES

1. **von Eiff C, Heilmann C, Proctor RA, Woltz C, Peters G, Götz F.** 1997. A site-directed *Staphylococcus aureus* hemB mutant is a small-colony variant which persists intracellularly. J. Bacteriol. **179:**4706-4712.
2. **Proctor RA, van Langevelde P, Kristjansson M, Maslow JN, Arbeit RD.** 1995. Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. Clin. Infect. Dis. **20:**95-102.
3. **Schaaff F, Bierbaum G, Baumert N, Bartmann P, Sahl HG.** 2003. Mutations are involved in emergence of aminoglycoside-induced small colony variants of *Staphylococcus aureus.* Int. J. Med. Microbiol. **293:**427-435.
4. **Abu-Qatouseh LF, Chinni SV, Seggewiß J, Proctor RA, Brosius J, Rozhdestvensky TS, Peters G, von Eiff C, Becker K.** 2010. Identification of differentially expressed small non-protein-coding RNAs in *Staphylococcus aureus* displaying both the normal and the small-colony variant phenotype. J. Mol.Med. **88:**565-575.
5. **Tuchscherr L, Heitmann V, Hussain M, Viemann D, Roth J, von Eiff C, Peters G, Becker K, Löffler B.** 2010. *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. J. Infect. Dis. **202:**1031-1040.
6. **Latimer J, Forbes S, McBain AJ.** 2012. Attenuated virulence and biofilm formation in *Staphylococcus aureus* following sublethal exposure to triclosan. Antimicrob. Agents Chemother. **56:**3092-3100.
7. **Brouillette E, Martinez A, Boyll BJ, Allen NE, Malouin F.** 2004. Persistence of a Staphylococcus aureus small-colony variant under antibiotic pressure in vivo. FEMS Immunol. Med. Microbiol. **41:**35-41.

8. **Gläser R, Becker K, von Eiff C, Meyer-Hoffert U, Harder J.** 2014. Decreased Susceptibility of *Staphylococcus aureus* small colony variants (SCVs) towards human antimicrobial peptides. J. Investig. Dermatol. **134**:2347-2350.
9. **Kahl B, Herrmann M, Everding AS, Koch HG, Becker K, Harms E, Proctor RA, Peters G.** 1998. Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis. J. Infect. Dis. **177:**1023-1029.
10. **Sendi P, Rohrbach M, Graber P, Frei R, Ochsner PE, Zimmerli W.** 2006. *Staphylococcus aureus* small colony variants in prosthetic joint infection. Clin. Infect. Dis. **43:**961-967.
11. **Lannergård J, von Eiff C, Sander G, Cordes T, Seggewiβ J, Peters G, Proctor RA, Becker K, Hughes D.** 2008. Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **52:**4017-4022.
12. **von Eiff C, McNamara P, Becker K, Bates D, Lei X-H, Ziman M, Bochner BR, Peters G, Proctor RA.** 2006. Phenotype microarray profiling of *Staphylococcus aureus* menD and hemB mutants with the small-colony-variant phenotype. J. Bacteriol. **188:**687-693.
13. **Kahl BC, Belling G, Reichelt R, Herrmann M, Proctor RA, Peters G.** 2003. Thymidine-dependent small-colony variants of *Staphylococcus aureus* exhibit gross morphological and ultrastructural changes consistent with impaired cell separation. J. Clin. Microbiol. **41:**410-413.
14. **Von Eiff C, Proctor RA, Peters G.** 2000. Small colony variants of Staphylococci: a link to persistent infections. Berl. Munch. tierarztl. Wochenschr. **113:**321-325.
15. **Besier S, Ludwig A, Ohlsen K, Brade V, Wichelhaus TA.** 2007. Molecular analysis of the thymidine-auxotrophic small colony variant phenotype of *Staphylococcus aureus*. Int. J. Med. Microbiol.**297:**217-225.
16. **Besier S, Ludwig A, Ohlsen K, Brade V, Wichelhaus TA.** 2007. Molecular analysis of the thymidine-auxotrophic small colony variant phenotype of *Staphylococcus aureus*. Int J Med Microbiol **297:**217-225.
17. **Schnitzer RJ, Camagni LJ, Buck M.** 1943. Resistance of small colony variants (G-forms) of a Staphylococcus towards the bacteriostatic activity of penicillin. Exp. Biol. Med. **53:**75-78.
18. **Gilman S, Saunders VA.** 1986. Accumulation of gentamicin by S*taphylococcus aureus*: The role of the transmembrane electrical potential. J. Antimicrob. Chemother. **17:**37-44.
19. **Besier S, Zander J, Kahl BC, Kraiczy P, Brade V, Wichelhaus TA.** 2008. The thymidine-dependent small-colony-variant phenotype is associated with hypermutability and antibiotic resistance in clinical *Staphylococcus aureus* isolates. Antimicrob. Agents Chemother. **52:**2183-2189.
20. **Balwit JM, Van Langevelde P, Vann JM, Proctor RA.** 1994. Gentamicin-resistant menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells. J. Infect. Dis. **170:**1033-1037.
21. **Nguyen HA, Denis O, Vergison A, Theunis A, Tulkens PM, Struelens MJ, Van Bambeke F.** 2009. Intracellular activity of antibiotics in a model of human THP-1 macrophages infected by a *Staphylococcus aureus* small-colony variant strain isolated from a cystic fibrosis patient: pharmacodynamic evaluation and comparison with isogenic normal-phenotype and revertant strains. Antimicrob. Agents Chemother. **53:**1434-1442.

22. **Kohler C, von Eiff C, Peters G, Proctor RA, Hecker M, Engelmann S.** 2003. Physiological characterization of a heme-deficient mutant of *Staphylococcus aureus* by a proteomic approach. J. Bacteriol. **185:**6928-6937.
23. **Rozen DE, McGee L, Levin BR, Klugman KP.** 2007. Fitness Costs of Fluoroquinolone Resistance in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **51:**412-416.
24. **Levin BR, Perrot V, Walker N.** 2000. Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. Genetics. **154:**985-997.
25. **Bayston R, Ashraf W, Smith T.** 2007. Triclosan resistance in methicillin-resistant *Staphylococcus aureus* expressed as small colony variants: a novel mode of evasion of susceptibility to antiseptics. **59:**848-853.
26. **Seaman PF, Ochs D, Day MJ.** 2007. Small-colony variants: a novel mechanism for triclosan resistance in methicillin-resistant *Staphylococcus aureus*. J. Antimicrob. Chemother. **59:**43-50.
27. **Barbolt TA.** 2002. Chemistry and safety of triclosan, and its use as an antimicrobial coating on Coated VICRYL* Plus Antibacterial Suture (coated polyglactin 910 suture with triclosan). Surg. Infect. **3:**s45-s53.
28. **Moran J, Addy M, Newcombe R, Marlow I.** 2001. A study to assess the plaque inhibitory action of a newly formulated triclosan toothpaste.J. Clin. Periodontal. **28:**86-89.
29. **Faoagali J, Fong J, George N, Mahoney P, O'Rourke V.** 1995. Comparison of the immediate, residual, and cumulative antibacterial effects of Novaderm R, Novascrub R, Betadine Surgical Scrub, Hibiclens, and liquid soap. Am. J. Infect. Control. **23:**337-343.
30. **Levy CW, Roujeinikova A, Sedelnikova S, Baker PJ, Stuitje AR, Slabas AR, Rice DW, Rafferty JB.** 1999. Molecular basis of triclosan activity. Nature. **398:**383-384.
31. **McMurry LM, Oethinger M, Levy SB.** 1998. Triclosan targets lipid synthesis. Nature **394:**531-532.
32. **Heath RJ, Rock CO.** 2000. Microbiology: a triclosan-resistant bacterial enzyme. Nature **406:**145-146.
33. **Villalain J, Mateo CR, Aranda FJ, Shapiro S, Micol V.** 2001. Membranotropic effects of the antibacterial agent Triclosan. Arch. Biochem. Biophys. **390:**128-136.
34. **Ledder RG, Gilbert P, Willis C, McBain AJ.** 2006. Effects of chronic triclosan exposure upon the antimicrobial susceptibility of 40 ex-situ environmental and human isolates. J. Appl. Microbiol. **100:**1132-1140.
35. **Seaman PF, Ochs D, Day MJ.** 2007. Small-colony variants: a novel mechanism for triclosan resistance in methicillin-resistant *Staphylococcus aureus.* J Antimicrob Chemother **59:**43-50.
36. **McMurry LM.** 1998. Triclosan targets lipid synthesis. Nature **394:**531.
37. **McMurry LM, McMurry.** 1998. Overexpression of marA, soxS, or acrAB produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*. FEMS Microbiol. Lett. **166:**305.
38. **Sanchez P.** 2005. The biocide triclosan selects *Stenotrophomonas maltophilia* mutants that overproduce the SmeDEF multidrug efflux pump. Antimicrob. Agents and Chemother. **49:**781.
39. **Bayston R, Ashraf W, Smith T.** 2007. Triclosan resistance in methicillin-resistant *Staphylococcus aureus* expressed as small colony variants: a novel mode of evasion of susceptibility to antiseptics. J. Antimicrob. Chemother. **59:**848-853.

40. **Moore LE, Ledder RG, Gilbert P, McBain AJ.** 2008. *In vitro* study of the effect of cationic biocides on bacterial population dynamics and susceptibility. Appl. Environ. Microbiol. **74:**4825.
41. **Andrews JM, Testing BWPoS.** 2001. BSAC standardized disc susceptibility testing method. J. Antimicrob. Chemother. **48:**43-57.
42. **Paulander W, Varming AN, Bæk KT, Haaber J, Frees D, Ingmer H.** 2012. Antibiotic-mediated selection of quorum-sensing-negative *Staphylococcus aureus.* MBio **3:**e00459-00412.
43. **Margolis PS.** 2000. Peptide deformylase in *Staphylococcus aureus:* resistance to inhibition is mediated by mutations in the formyltransferase gene. Antimicrob. Agents Chemother. **44:**1825.
44. **Stapleton MR.** 2007. Characterization of IsaA and SceD, two putative lytic transglycosylases of *Staphylococcus aureus.* J. Bacteriol. **189:**7316.
45. **Chuard C, Vaudaux PE, Proctor RA, Lew DP.** 1997. Decreased susceptibility to antibiotic killing of a stable small colony variant of *Staphylococcus aureus* in fluid phase and on fibronectin-coated surfaces. J. Antimicrob. Chemother. **39:**603-608.
46. **Kunz AN, Begum AA, Wu H, D'Ambrozio JA, Robinson JM, Shafer WM, Bash MC, Jerse AE.** 2012. Impact of fluoroquinolone resistance mutations on gonococcal fitness and *in vivo* selection for compensatory mutations. J. Infect. Dis.**205:**1821-1829.
47. **Andersson DI, Levin BR.** 1999. The biological cost of antibiotic resistance. Curr. Opin. Microbiol. **2:**489-493.
48. **Guo B, Abdelraouf K, Ledesma KR, Nikolaou M, Tam VH.** 2012. Predicting bacterial fitness cost associated with drug resistance. J. Antimicrob. Chemother. **67:**928-932.

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).

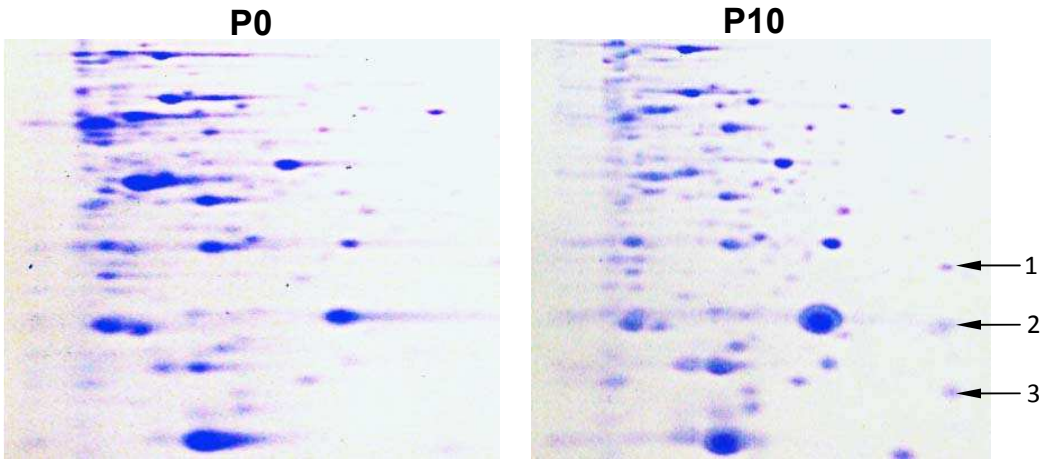
558

559 **FIG. 1**

560

561

A



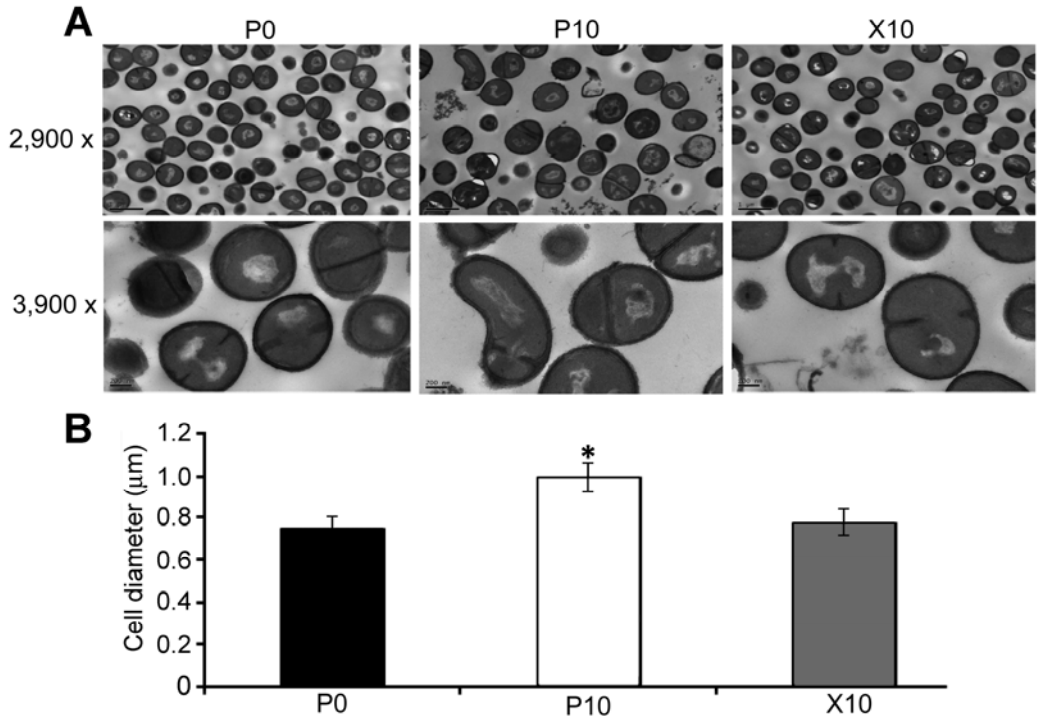
B

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



563
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

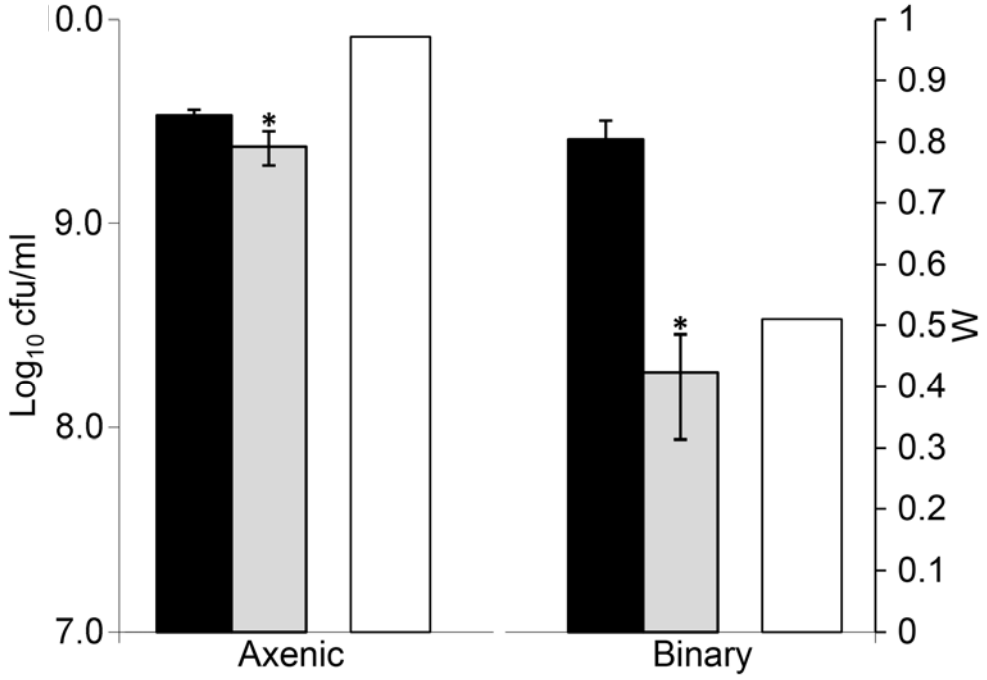


FIG 3. Competitive fitness of P0 *vs* P10 (SCV) in *S. aureus* ATCC 6538. Black and grey bars show cfu/ml of P0 and P10, respectively after 24h of growth, axenically or in binary culture. White bars indicate relative fitness (W) under axenic and binary growth. Data are means and standard deviations from 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).